BIOCHEMICAL AND GENETIC CHARACTERIZATION OF BACTERIA ISOLATED FROM DISEASED RAINBOW TROUT (*Oncorhynchus mykiss*) FARMED IN LESOTHO AND MPUMALANGA PROVINCE OF SOUTH AFRICA

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Thesis presented in fulfillment of the requirements for the degree of

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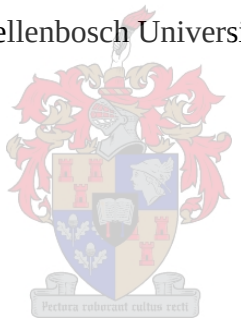

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December 2017

DECLARATION

By submitting this thesis, I therefore declare that the work contained within is my own original work as assisted by my supervisors, that I am the owner of the copyright thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any other qualification.

Ms Vuyokazi Kutu

Date: December 2017

ABSTRACT

Rainbow trout farms in Mpumalanga Province, South Africa and Lesotho, have periodically suffered significant losses from infections caused by Gram-positive bacteria. Such outbreaks have hampered the development of this industry in both South Africa and Lesotho. A total of 55 bacterial strains had been isolated between 2006-2012 from infected trout farmed in Lesotho and Mpumalanga Province and had been stored for long term by freeze drying. Some isolate identification had been performed and a few were used for vaccine development. Vaccines were however only effective for one or two seasons, highlighting the need to properly characterize these Gram-positive bacteria. The aims of the study were therefore to: (i) investigate the genetic diversity of these bacterial isolates by their phenotype; antimicrobial susceptibility and 16S ribosomal RNA (rRNA) sequencing and phylogenetic analysis, (ii) investigate the different antigenic epitopes that exist within this group of bacterial isolates by development of an enzyme-linked immunosorbent assay (ELISA) utilizing six rabbit produced polyclonal antibodies, produced against six selected bacterial isolates from the 55 isolates investigated in this study.

Phenotypic analysis showed that fifty of the isolates were Gram-positive cocci and five were Gram-positive rods. Their growth characterists and antimicrobial susceptibility were extensively characterized. The 16S rRNA analysis indicated the following isolate composition: 49 *Lactococcus garvieae*, one *Lactococcus lactis*, three *Carnobacterium maltaromaticum* and two *Weissella* species, which is the first report of *Weissella* from diseased trout from South Africa.

Antigenicity analysis showed that there were highly specific epitopes that were limited to very few isolates, but also common epitopes that were shared between isolates of the same genus, but even some epitopes that were shared between different bacterial genera. The patterns of epitope sharing broadly correlated with the 16S rRNA phylogeny, but not entirely which was not unexpected as phylogeny does not indicate the presence or absence of bacterial epitopes.

These results address the importance and accuracy of molecular identification of disease causing species and the need to investigate the antigenic differences expressed by these pathogenic bacteria to assist in generating correct information needed for the development of vaccines of high efficacy.

OPSOMMING

Reenboogforelplase in Mpumalanga, Suid-Afrika, en Lesotho het met tussenposes betekenisvolle verliese gelei as gevolg van infeksies veroorsaak deur Gram-positiewe bakterieë. Sulke uitbrake het die onwikkeling van die bedryf beperk in sowel Suid-Afrika asook Lesotho. ‘n Totaal van 55 bakteriese rasse is geïsoleer tussen 2006-2012 van geïnfekteered forelle waarmee geboer is in Lesotho en Mpumalanga en wat langtermyn deur vriesdroging opgeberg is. Party isolate is geïdentifiseer en ’paar is vir entstofontwikkeling gebruik. Entstowwe was egter slegs vir een of twee seisoene effektief, wat die belang van die karakterisering van die Gram-positiewe bakterieë beklemtoon het. Die doelwitte van hierdie studie was dus om: (i) die genetiese diversiteit van die bakteriele isolate deur fenotipe; antimikrobiese gevoeligheid en 16S ribosomale RNA (rRNA) volgordebepaling en filogenetiese analise te bepaal, (ii) die verskillende antigeniese epitope wat binne hierdie groep voorkom, deur middel van ensiem gekoppelde immunoessais (ELISA) wat ontwikkel is met ses konyn verwekte poliklonale antiliggame wat teen ses geselekteerde bakteriele isolate geproduseer is, te bepaal.

Fenotipiese analise van vyftig van die isolate is gevind om Gram-positiewe cocci en vyf om Gram-positiewe stawe te wees. Hulle groei eienskappe en antimikrobiese gevoeligheid is deeglik gekarateriseer. Die 16S rRNA analise het die volgende isolaat samestelling aangedui: 49 *Lactococcus garvieae*, een *Lactococcus lactis*, drie *Carnobacterium maltaromaticum* en twee *Weissella* spesies, wat die eerste berig is van die voorkoms van *Weissella* in Suid-Afrikaanse forelle.

Antigeniese analise het getoon dat hoogs spesifieke epitope beperk is tot sekere isolate, maar dat daar ook ‘n paar epitope voorkom wat deur isolate gedeel word tussen lede van dieselfde genus, en selfs tussen verskillende bakteriele genera. Die patrone van epitoop deling korreleer in die bree met die 16S rRNA filogenie, maar nie heeltemal nie, wat nie onverwag was nie, aangesien filogenie nie die teenwoordigheid of afwesigheid van epitope kan aandui nie.

Hierdie resultate beklemtoon die belang en akkuraatheid van molekulêre identifikasie van siekteverwekkende spesies en die behoefte om die antigeniese verskille tussen hierdie patogeniese bakterieë te ondersoek om korrekte inligting te genereer vir die ontwikkeling van

hoogs doeltreffende entstowwe.

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DEDICATIONS

I dedicate this thesis to my family: my mother Bulelwa Doris Kutu, my father Sivuyile Owen Kutu, my late brother Siphamandla Kutu and my sister Zamacirha Kutu.

I also dedicate this thesis to Dr Brett Macey, for allowing me to work on this project with him, for his support, for never giving up on me and for his never-ending dedication to excellence.

ABBREVIATIONS

Ab	Antibody
ANOVA	Analysis of variance
Ag	Antigen
ABTS	2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)
bp	base pairs
BA	blood Agar
CFU	Colony Forming Units
DNA	Deoxyribonucleic acid
dNTP	Deoxy-ribonucleoside triphosphate
dH_2O	Distilled water
ELISA	enzyme-linked immunosorbent assay
EDTA	ethylene-diamenetetra-acetic acid
IgB	biotinylated antibodies
hrs	Hours
Ig	immunoglobulin
IP	intraperitoneal
IM	Intramuscular
kb	kilobase(s)
LA	Luria agar
LB	Luria broth
LSO	Lesotho
LPS	lipopolysaccharide
min	minutes
M	molar

mM	Milli molar
MAP	modified atmosphere packaged
NCBI	National Center for Biotechnology Information
NA	Nutrient agar
O/N	over night
OD	Optical Density
PG	Penicillin G
PAUP*	Phylogenetic analysis using parsimony
PBS	Phosphate-buffered saline
PCR	polymerase chain reaction
PLG	polylactide-co-glycolide
rDNA	ribosomal DNA
rRNA	ribosomal RNA
RPS	relevance percentage survival
rpm	revolutions per minute
sp.	species
spp.	subspecies
SEM	Standard error of mean
TAE	Tris-acetate EDTA
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
v/v	volume/volume
w/v	Weight/volume

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CHAPTER 1: GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 Aquaculture: global and local status

Aquaculture is one of the fastest growing animal production sectors in the world, with growth in global cultured fish production increasing substantially in the last five decades (FAO, 2014). As such, this sector is expected to be the lead player in achieving long-term food and nutrition security, meeting the increased demand for fish food through continued promotion of sustainable aquaculture development. High population growth in the 20th century combined with an increased average household income and high consumer demand for more nutritious and higher quality foods has increased the demand for seafood products, which are rich in micronutrients and contain high levels of omega-3 fatty acids (Gjedrem et al., 2012). Freshwater fish farming makes the greatest direct contribution to the supply of affordable protein food, accounting for about 17% of the global population's intake of animal protein (FAO, 2014). Since the high demand for seafood products can no longer be provided for by capture fisheries, aquaculture is regarded as the solution to help supplement wild stocks and sustain a growing demand for both freshwater and marine organisms, which are currently over exploited (FAO, 2014; C & R, 2014). World aquaculture fish food production has increased by an average of 6.2% between the year 2000 and 2012. Production further increased by 5.8% from 66.6 million tons in 2012 (worth US$ 137.7 billion) to 70.5 million tons in 2013 (FAO, 2014).

The South African aquaculture industry has expanded moderately over the last few years with an estimated fish food production of above 5200 tons in 2014, excluding the contribution from seeweeds and ornamentals (South African Aquaculture Yearbook, 2014). Currently, South African aquaculture contributes approximately 0.2% (R 0.7 billion) to the country's gross domestic product (GDP) and a contribution of 0.002 million tons (0.00003%) of the estimated global production, which is approximately 67 million tons (Operation Phakisa, 2014). Even though South African fish consumption is estimated to increase at a very low rate as compared to the rest of the world, it is however expected to contribute more to food safety, sustainable job creation, economic development and foreign investments (South African Aquaculture Yearbook, 2014).

1.2 Trout farming in South Africa and Lesotho

Trout: rainbow trout (*Oncorhynchus mykiss*) and brown trout (*Salmo trutta*) were initially introduced to South Africa for angling purposes in 1897 (Salmoniformes et al., 2012; Safriel & Bruton, 1984). Being the oldest aquaculture subsector in South Africa (Shipton & Britz, 2007), trout is the most cultured freshwater species in South Africa with farms located in the Western Cape, Eastern Cape, Kwa-Zulu Natal and Mpumalanga (Fig. 1.1; CD: Aquaculture and Economic Development, 2014). Trout production in South Africa has increased from approximately 800 tons per annum in 2006 to over 1600 tons in 2013, making it one of the largest aquaculture sub-sectors in the region (Fig. 1.2).

Figure 1.1: Map representing trout facilities within South Africa, courtesy of Johan du Plessis, Chief Directorate: Aquaculture and Economic Development, 2014.

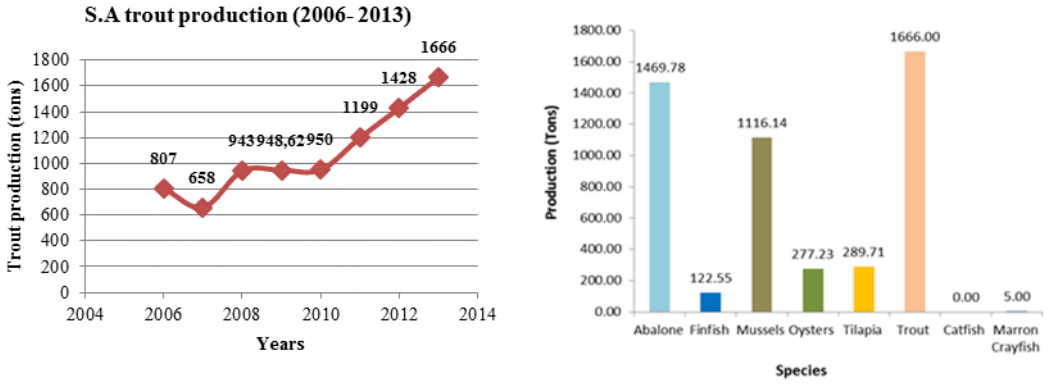

Figure 1.2: South African trout production from 2006-2013 and South African aquaculture production per sub-sector for the year 2013 (CD:AED, 2014).

Lesotho is a small land locked country, geographically surrounded by South Africa, located between 28 º and 31 ºS latitude and 27 º and 30 ºE longitude (Area= 30,355 km^2). With a population of 1.88 million people, about 80% of the country's population is dependent on the agricultural sector for their livelihoods, with fish farming, trout farming in particular, playing a significant role (US Department of Commerce, 2013). Rainbow trout (*O. mykiss*) farming began in 2006 at the Lesotho Katse dam as part of the Lesotho Highlands Water Project (fig 1.3(a), with production of about 300 tons recorded in 2007, which was then estimated (www.highlandstrout.co.za; (FAO, 2008) at 800 tons in 2013 (US Department of Commerce, 2013). No other estimates have been published recently but, the current data gave an estimated 333 950 kg in 2015 (fig 1.3(b)) (courtesy of Ed Studdom, 2016 of Katse dam project).

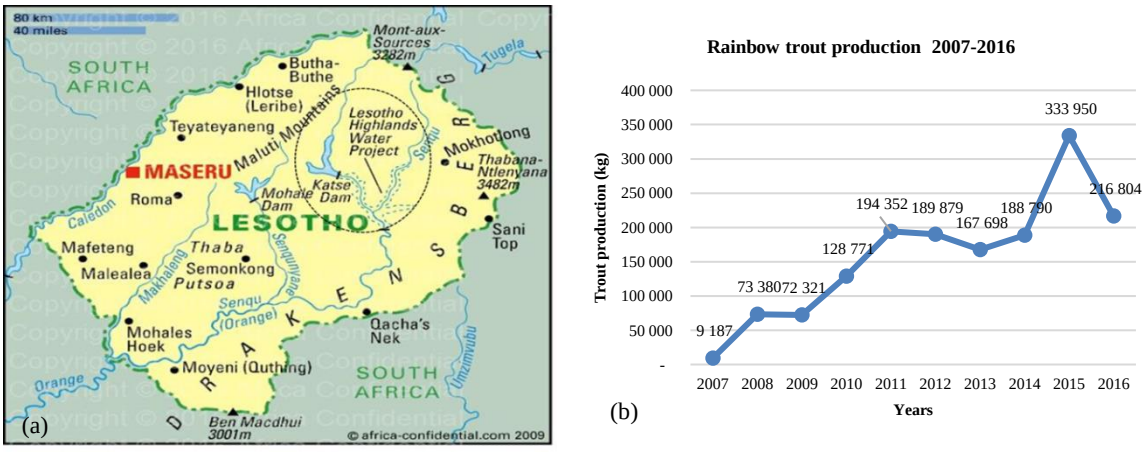

Figure 1.3: (a) The Kingdom of Lesotho showing Katse dam where rainbow trout farming mostly occurs. (b) Rainbow trout production (in kilograms) from Lesotho from 2007-2016 (courtesy of Ed Studdom).

1.3 Streptococcosis

Streptococcosis is a bacterial disease that affects both captive and wild populations of freshwater and marine fish species throughout the world (Al-Harbi, 2011). The disease is considered one of the main limiting factors in the global trout aquaculture industry and has caused significant economic losses to date, resulting in an estimated annual loss of US$ 150 million in 2000, which increased to US$ 250 million in 2008 (Amal & Zamri-Saad, 2011). The first reported streptococcosis outbreak in cultured fish was reported in rainbow trout (*O. mykiss*) from Japan by Hoshina et al. (1958) (Suanyuk & Itsaro, 2011). Over the years, more and more streptococcal infections have been identified as the cause of mortality and morbidity in both farmed and captured fish worldwide (Agnew & Barnes, 2007). In marine fish, Streptococcosis has been reported from a variety of commercially imported species, such as yellowtail (*Seriola spp*.), striped mullet (*Mugil cephalus*), eel (*Anguilla japonicas*), striped bass (*Morone saxatilis*), sardine (*Sardinops melanostictus*) and sea catfish (*Arius felis*) (Kusuda & Salati, 1999). In the freshwater environment, Streptococcosis has been reported from fish species such as rainbow trout (*O. mykiss*) and tilapia (*Tilapia nilotica*) (Ferguson et al., 1994).

1.3.1 Etiology

Streptococcosis is caused by *Streptococcus* species such as: *S. agalactieae, S. iniae, S. parauberis, S. ictaluri* and *S. mileri*. However, several closely related Gram-positive bacterial species have been isolated and identified from fish exhibiting similar clinical signs as Streptococcosis, including bacteria that belong to Order Lactobacillales and the family Streptococcaceae (genera *Streptococcus, Lactococcus*), family Carnobacteriaceae (genus *Carnobacterium*), family Leuconostocaceae (genus *Weissella*), and family Enterococcaceae (genus *Vagococcus* and *Enterococcus*) (Buller, 2014; Ruiz-Zarzuela et al., 2005; Kia & Mehrabi, 2013). These bacteria are classified as lactic acid bacteria (LAB), meaning that they produce lactic acid as the main end product of carbohydrate fermentation (Ringø & Gatesoupe, 1998; Afzal & Lorraine, 2014). This disease is termed according to the causative agent as described in Table 1.1. For instance, it is referred to as Streptococcosis if caused by any of the *Streptococcus* species, lactococcosis when caused by *Lactococcus* species (*L. garvieae, L. lactis, L. lactis subs lactis*) (Pridgeon & Klesius, 2014), vagococcosis if caused by *Vagococcus* species enterococcosis if caused by *Enterococcus* species (Kusuda & Salati, 1999) and weisselliosis when caused by *Weissella* species (Welch, 2014).

Table 1.1: List of Gram-positive bacteria that cause disease similar to Streptococcosis in rainbow trout (Buller, 2014).

Host	**Phylum:** Firmicutes **Class:** Bacilli **Order:** Lactobacillales			
Rainbow trout (*O. mykiss* Walbaum 1792)	**Genus**	**Pathogen**	**Clinical signs and tissue site**	**Disease status**
	Family: Carnobacteriaceae			
	Carnobacterium (rods)	*Carnobacterium maltaromaticum*	Bilateral exophthalmia, periocular haemorrhages, ascites fluid and haemorrhages in liver, swim bladder, muscle and intestine, inflammation in the brain, kidney.	Disease termed Pseudo-kidney disease, chronic disease with low level mortality associated with stress such as spawning.
	Family: Streptococcaceae			
	Lactococcus (coccus)	*L. garvieae*	Haemorrhagic septiceamia, Exophthalmos	Mortality
		L. lactis spp. *tructae*	Normal flora of intestinal mucus	Normal flora
		L. piscium		Pseudo-kidney disease, lactobacillosis
	Streptococcus (coccus)	*S. agalactiae*	Organisms in brain and eye, erratic swimming, exophthalmia, haemorrhagic around the body.	Meningoencephalitis, septicaemia.
		S. iniae	Organisms in brain and eye	Meningoencephalitis, septicaemia.
	Family Enterococcaceae			
	Vagococcus (rods)	*Vagococcus salmoninarum*	Loss of equilibrium, haemorrhage around eye and gill, lesions on body, congestion in spleen and liver. Peritonitis, haemorrhagic ascites, retained eggs, engorged testes, languid swimming. Pure cultures best collected from eye, brain and kidney.	Vagococcosis, chronic disease with mortality.
	Family Leuconostocaceae			
	Weissella (rods)	*Weissella* species	Brain, kidney, liver	Haemorrhagic septiceamia

1.3.2 Clinical manifestation, signs and symptoms

One of the major factors that predispose fish to a disease such as Streptococcosis is stress. Stress is caused by a number of biological and physical factors (Malham et al., 2003). Biological factors include high stocking densities and the associated competition for space;

food quality and quantity; and the presence of opportunistic pathogens. Physical factors include poor water quality, such as increased levels of pH, ammonium and decreased oxygen levels or dissolved gas pressure; trauma experienced by fish when handled during spawning or harvesting periods; and the presence of chemical pollutants in the water. Both biological and physical stress have been shown to compromise fish immunity and render them susceptible to opportunistic pathogens (Huchzermeyer, 2003; Huchzemeyer & Henton, 2011; Burnett et al., 2007; Alvarez-Pellitero, 2008). The main environmental factor associated with outbreaks of Streptococcosis in cultured fish is changes in water temperature. Warm water infections occur at temperatures above 15 ºC and are generally caused by pathogenic Gram-positive bacteria such as *S. iniae*, *S. parauberis* and *L. garvieae*, which are pathogenic for both cultured marine and freshwater fish species (Vendrell et al., 2006). Conversely, cold water infections occur at temperatures below 15 °C and are caused by bacteria such as *V. salmoninarum* and *L. piscium* (Vendrell et al., 2006). In South Africa, warm water streptococcal infections have predominantly been reported to occur at temperatures above 19 °C (Boomker et al., 1979). As classic cold water conditions do not occur in South Africa, the Family Enterococcaceae are not discussed further in this literature review.

The kidneys, brain and the liver are the main organs from which Gram-positive bacteria have been isolated. Clinical signs of this disease include exophthalmos on either one or both eyes, lethargy, anorexia due to loss of appetite, dark coloration of the skin, and congestion of the pectoral and caudal fins and mouth (Boomker et al., 1979). Internally, the spleen is often enlarged, the liver is pale and there is inflammation around the kidney and heart (Yanong & Francis-floyd, 2010; Vendrell et al., 2007). Infected fish tend to swim erratically as the causative pathogen invades the brain and the nervous system (Yanong & Francis-floyd, 2010; Huchzermeyer, 2011).

1.4 Bacteria responsible for Streptococcosis

1.4.1 Family Streptococcaceae:

1.4.1.1 Genus *Streptococcus*

1.4.1.1.1 Morphology and culture

Streptococcus species are non-motile, Gram-positive, non-spore forming cocci that occur in pairs or chains of varying length. Most bacteria belonging to this genus are oxidase negative and lack the enzyme catalase, are facultative anaerobes and may require the addition

of CO_2 for growth. During carbohydrate fermentation, *Streptococcus* species produce lactic acid, ethanol, acetic acid and formic acid. However, one strain, *S. didelphis*, has been shown to be catalase positive when grown on blood agar medium, but loses this capacity during subsequent sub-culturing. The genus *Streptococcus* has about 20 antigenic groups that have been identified to date, designated A to H or K to V and these are termed Lancefield groups (Center for Food Security & Public Health, 2005). However, the Lancefield grouping is no longer as effective as there are some newly described *Streptococcus* species that do not have any Lancefield antigens. One of the main challenges in grouping some of these *Streptococcus* species is the fact that in some cases members of the same species may belong to more than one Lancefield group (Center for Food Security & Public Health, 2005). Characterization is also based on ability to break down red blood cells (haemolysis) in a blood agar medium. There are three types of haemolysis: Beta-haemolysis, Alpha-haemolysis and Gamma-haemolysis. *S. iniae*, in particular, is a beta-haemolytic bacterium that is well known for its ability to completely lyse red blood cells on a blood agar plate. Other *Streptococcal* species are classified to be either alpha-haemolytic, based on their inability to lyse red blood cells, or gamma-haemolytic, where minimal hydrolysis of red blood cells occurs. Since around the 1980s, the Streptococci have been reclassified to *Streptococcus*, *Lactococcus* and *Enterococcus* based not only on their cell carbohydrate antigens and haemolytic characteristics but several other phenotypic characteristics (Facklam, 2002).

1.4.1.1.2 Epidemiology

Several species of *Streptococcus* have been implicated as the causative agents of Streptococcosis of fish, including *S. iniae*, *S. faecalis, S. uberis, S. faecium, S. agalactiae, S. parauberis, S. ictaluri*, *S. dysgalactiae*, and *S. phoae*, to name a few (Pourgholan et al., 2011). Streptococcal infections of rainbow trout have been studied in several countries and endocarditis, meningitis, cellulitis and septic arthritis are among the listed diseases caused by *S. iniae* infections in humans (Sun et al., 2010).

1.4.1.1.3 Pathogenicity in fish

Pathogenicity differs among *Streptococcus* species. Hemolysins are known exotoxins involved in the pathogenicity of *Streptococcus* species, with Streptolysin S and O being the most common exotoxins identified to date. Other streptococcal species, such as *S. equi, S. pyogenes* and *S. dysgalactiae*, possess M and M-like proteins that are located on the surface of the bacterial cell wall (Baiano et al., 2008). These proteins are considered to be the most

frequent virulence factors involved in host-cell attachment and anti-phagocytic activity (Locke et al., 2007).

The vast majority of published research on the pathogenicity of streptococcal species has focused on *S. iniae*. The first *S. iniae* infection was reported in 1976 at San Francisco from an Amazon freshwater dolphin (*Inia geoffransis*) with 'golf ball' disease (Pier & Madin, 1976). Since then, several cases of *S. iniae* infections have been reported globally. *S. iniae* is a pathogenic bacterium that not only affects a wide range of freshwater and marine fish, but has been isolated from disease cases in humans and other terrestrial animals and is hence regarded as a zoonotic pathogen (Agnew & Barnes, 2007). Miller & Neely (2005) demonstrated that *S. iniae* mutants, which have a capsule, were more resistant to phagocytosis, indicating the importance of the capsule for pathogenicity and successful infection of the host. The fibrinogen binding cell-surface proteins of *S. iniae* have also been shown to be important for avoiding phagocytic attack by host macrophages (Baiano et al., 2008). The phosphoglucomutase enzyme, which converts glucose-1-phosphate to glucose-6-phosphate in the glycolytic pathway and which also plays a role in producing *S. iniae* polysaccharide capsules, has also been identified by Buchanan et al. (2005) to be one of the virulence factors produced by *S. iniae*.

1.4.1.2 Genus *Lactococcus*

1.4.1.2.1 Morphology and culture

Lactococcus species are Gram-positive cocci that occur either as single cells or, in pairs or in short chains. Bacteria belonging to this genus are non-motile, produce no endospores, and are catalase and oxidase negative facultative anaerobes (Vendrell et al., 2006; Fihman et al., 2006) that grow at 10 °C, but not at 45 °C (Bergey's manual, 2011).

1.4.1.2.2 Epidemiology

Several species have been identified under this genus: *L. lactis* ssp. *lactis, L. lactis* ssp. *tructae, L. lactis* ssp. *hardniae, L. lactis* ssp. *cremoris, L. garvieae* (previously known as *S. garvieae* (Buller, 2014), *L. piscium, L. plantarum, L. raffinolactis, L. chungangensis, and L. fujiensis* (Miyauchi et al., 2012). Of the latter mentioned organisms, *L. lactis* and *L. garvieae* are the most pathogenic species that have been reported and identified most frequently to date and both of these species have caused major problems in the aquaculture industry (Aguado-Urda et al., 2011; Miyauchi et al., 2012). *L. garvieae* has been associated with numerous

cases of disease, not only in fish, but in humans and other wild and farmed terrestrial animals, including several cases of subclinical mastitis in cows and water buffalo (Balca et al., 2006). In humans, this bacterium has been isolated from blood samples, skin and urinary tract clinical specimens obtained from patients suffering from bacterial endocarditis (Vendrell et al. 2006). *L. garvieae* has also been isolated from various kinds of dairy products, such as cheese, raw cow's milk, vegetables and poultry meat, hence it is considered a potentially opportunistic zoonotic pathogen (Vendrell et al., 2006; Miyauchi et al., 2012).

Lactococcosis refers to the disease Streptococcosis that is caused by *Lactococcus* species. The first outbreak of this disease in rainbow trout was reported from Spain in 1988 (Ghittino & Prearo, 1992). Since then, *L. garvieae* has caused severe mortalities in the rainbow trout sector in many countries worldwide (Tanrikul & Gultepe, 2011). Other fish species that have been diagnosed with *L. garvieae* infections include Japanese eel (*Anguilla japonicas*), yellowtail *(Seriola quinqueradiata*) (Maki et al., 2008) and black rockfish (*Sebastes schlegeli*) (Vendrell et al., 2006), however, the highest economic impact has been in rainbow trout (*O. mykiss)* (Miyauchi et al., 2012; Vela et al., 2000; Eldar & Ghittino, 1999). Lactococcosis affects rainbow trout of all sizes, from juveniles of 5 g to adults weighing more than 1 kg (Vendrell et al., 2006; Chang et al., 2002; Pereira et al., 2004). In Turkey, *L. garvieae* was isolated for the first time in 2011 from diseased rainbow trout, where mortalities of approximately 80% were recorded. Outbreaks have also been reported from France, Portugal, Israel, England and Korea. There are however, species like common carp (*Cyprinus carpio*), that are resistant to the disease (Vendrell et al., 2006; Eldar et al., 1996).

1.4.1.2.3 Pathogenicity in fish

Although the pathogenic mechanism of *L. garvieae* is not well understood, several studies have demonstrated that a capsular polysaccharide on the cell wall surface plays a role in the virulence associated with fish pathogenic strains (Ferrario et al., 2012), especially its ability to agglutinate (Balca et al., 2006). A study by Morita et al. (2011) demonstrated that a sequence of genes involved in capsule formation by *L. garvieae* Lg2 had high similarities with a 16.5 kb gene cluster from *L. lactis*, which is responsible for capsule production. Since then, *L. garvieae* was serologically classified into two groups based on the presence or absence of this polysaccharide capsule: a capsulated/virulent group (serotype KG-) and a non-capsulated/virulent group (serotype KG+) (Morita et al., 2011).

1.4.2 Family Carnobacteriaceae

1.4.2.1 Genus *Carnobacterium*

1.5.2.1.1 Morphology and culture

Carnobacterium is a genus of non-motile and non-sporulating Gram-positive rod shaped bacteria, approximately 1.1-1.4 µm x 0.5-0.6 µm in size. The bacteria usually occur singularly or in pairs, and to a lesser extent in chains (Collins et al., 1987). Bacteria within this genus are facultative anaerobes, while some strains grow aerobically or microaerophilically (Afzal & Lorraine, 2014). *Carnobacterium* species can be differentiated from other lactic acid bacteria by their ability to produce L(+) lactate, the presence of meso-diaminopimelic acid in their cell wall (Scarpellini et al., 2002; Hamnes et al., 1991) and their ability to grow at pH 9.0, but not at pH 4.5 (Buller, 2014). Some species within this genus are considered to be psychrotolerant, growing at temperatures as low as 0 ºC, but cannot grow at above 45 ºC.

1.4.2.1.2 Epidemiology

Several species have been identified in the genus *Carnobacterium* (first proposed by Collins et al. (1987): *C. alterfunditum, C. divergens, C. funditum, C. gallinarum, C. inhibens, C. mobile, C.viridans, C. piscicola* and *C. pleistocenium.* Based on a phylogenetic analysis of the 16S and 23S rRNA genes, *C. piscicola* (also previously known as *Lactobacillus piscicola*) and *L. maltaromicus*, were both reclassified as *C. maltaromaticum* (Buller, 2014). *Carnobacterium* species have been isolated from a variety of sources, such as the human intestine, blood and skin as well as various food products including seafood, meat products and dairy products (Laursen et al., 2005). *Carnobacterium* has also been reported to be present as part of the intestinal microflora of Atlantic salmon and rainbow trout (Austin, 2006). *C. maltaromaticum* has also been isolated from the gills and intestine of healthy fish species, including Atlantic salmon (Joborn et al., 1997), hatchery-reared and wild stocks of Arctic charr, striped bass, channel catfish (Leisner et al., 2012) and rainbow trout (Ringø & Gatesoupe, 1998).

1.4.2.1.3 Pathogenicity in fish

Most *Carnobacterium* species are regarded as non-pathogenic, opportunistic pathogens considered to have low virulence characteristics with probiotic potential against other fish diseases (Starliper et al., 1992). However, *C. maltaromaticum* (previously known as *C. piscicola*) was isolated in Oregon (USA) in 1970 (Hiu et al., 1984) from diseased rainbow

trout (*O. mykiss*), cutthroat trout (*Salmo clarkii clarkii*) and spring chinook salmon (*O. tshawytscha*) (Buller, 2014). The disease caused by *Carnobacterium* in fish is called Pseudo-kidney disease and clinical symptoms observed from infected fish are similar to those associated with Streptococcosis. These symptoms include darkening of the skin; internal haemorrhage (liver, intestines, and swim bladder); lethargy; loss of appetite; accumulation of ascitic fluid; enlarged liver and spleen, as well as kidney damage. There is limited knowledge about the virulence factors involved during *Carnobacterium* infections in fish. However, a few studies have given great insight into the disease. Characteristic traits observed from *Carnobacterium* strains isolated from diseased fish include lack of haemolytic activity, as detected in *C. viridans*, and production of phospholipase and elastase activities, as described by Baya et al. (1991). Michel et al. (1986) observed that the majority of fish infected with *Carnobacterium* are adults that have experienced some form of stress, including handling of fish during farm production and stress associated with spawning (Buller, 2014). These authors concluded that stress predisposes fish to infection with these bacteria, as *Carnobacterium* infections are frequently associated with secondary invaders such as *Aeromonas hydrophila*. A case reported by Toranzo et al. (1993) indicated that the *C. maltaromaticum* strain (PT-31), that had caused an outbreak of Pseudo-kidney disease in market-size rainbow trout farmed in Spain, exhibited a high degree of virulence and the haemorrhage displayed by the infected fish was as severe as that caused by *Streptococcus* species. Furthermore, Leisner et al. (2012) studied the genes encoded by *C. maltaromaticum* that may assist the bacterium in becoming virulent in fish species and concluded that certain *C. maltaromaticum* strains exhibit virulence factors. Another study by Schaffer et al., (2013) on a meningoencephalitis case in juvenile salmon sharks (*Lamna ditropis*) revealed that *C. maltaromaticum*, through sequencing of the 16S rDNA gene, as the main cause of the disease.

1.4.3 Family Leuconostocaceae

1.4.3.1 Genus *Weissella*

1.4.3.1.1 Morphology and culture

Weissella species are Gram-positive, catalase-negative, non-spore forming non-motile coccoids or rods. Only one strain, *W. beninensis*, is reportedly motile (Padonou et al., 2010). *Weissella* species are obligate hetero-fermenters, meaning they ferment glucose via two pathways; the phosphoketolase pathway and hexose-monophosphate pathway, producing

ethanol or acetate, CO_2 and both D (-) and L (+) lactic acid. Bacteria belonging to this genus grow optimally at around 15 °C, while some strains can grow between 42–45 °C (*W. ceti* grows at 22-37 ºC, while *W. hellenica* grows at 15 - 40 °C). The mol% G+C content between strains differs from 37 to 47% (Figueiredo et al., 2011; Lonvaud-Funel, 2014).

1.4.3.1.2 Epidemiology

First proposed by Collins et al. (1993), this genus is grouped in five phylogenetic branches based on the 16S phylogeny comprising of 19 described species. Members of the first branch: *W. soli*, *W. diestrammenae*, *W. koreensis*, *W. kandleri*, and *W. oryzae*. Members of a second branch: *W. cibaria* and *W. confusa*. Members of the third branch: *W. thailandensis*, *W. hellenica* and *W. paramesenteroides*. *W. ceti*, *W. halotolerans*, *W. viridescens*, *W. minor*, and *W. uvarum* form the fourth branch, and *W. beninensis*, *W. fabalis*, *W. fabaria*, and *W. ghanensis* form the fifth branch (De Bruyne et al., 2010; Fusco et al., 2015). *Weissella* species are members of the Lactic acid bacterial group that belong to the class Bacilli, order Lactobacillales, family Leuconostocaceae (Björkroth et al., 2002; Fusco et al., 2015; Segalen & Oenon, 2014) and have been isolated from a variety of different sources, including plants and vegetables, fermented food (Björkroth et al., 2002), animal saliva, breast milk and the gastrointestinal tract of animals and humans (Lee et al., 2012b). *W. confusa*, *W. cibaria* and *W. viridescence* have been described as human opportunistic pathogens, causing infections such as bacteraemia (Lee et al., 2011) and endocarditis (Hase et al., 2015). *W. hellenica* has been isolated from the intestinal contents of flounder (*Paralichthys olivaceus*) (Vela et al., 2011; Björkroth et al., 2002).

1.4.3.1.3 Pathogenicity in fish

Infections caused by *Weissella* species in rainbow trout were first described from China in 2007 (Liu et al., 2009). Since then, mortalities on rainbow trout farms caused by *Weissella* species have been reported in Brazil (Figueiredo, 2012) and south-eastern United States (Welch & Good, 2013). These infections were reported to be similar to those caused by *Streptococcus* and *Lactococcus* bacteria as haemorrhagic septicemia. Symptoms include haemorrhage in the eyes and intestines, lethargy, anorexia, and exophthalmos. Bacteria have been isolated from organs such as the liver, kidney and the brain of infected fish. To the best of our knowledge, only one scientific paper has been published where a comparison and identification of putative virulence genes of these *Weissella* species infecting rainbow trout (Ladner et al., 2013) were performed. These authors analyzed genes from *W. ceti* NC36, a

representative strain from an outbreak of weisselliosis in the Unites States, and identified five collagen adhesins (WCNC_00912, WCNC_00917, WCNC_00922, WCNC_05547, and WCNC_06207), a mucus-binding protein (WCNC_01840) and a platelet associated adhesin (WCNC_01820). The source of these *Weissella* species is still unknown and their pathogenicity on rainbow trout is still unknown (Buller, 2014).

1.5 Control of bacterial diseases in aquaculture

As aquaculture production continues to expand and intensify worldwide, the emergence of highly virulent pathogens is expected to increase and as a consequence the importance of managing infectious diseases will increase as well. Economic losses imposed by outbreaks of infectious diseases are regarded as one of the major threats to the industry, with diseases caused by bacteria regarded as the most prevalent; constituting about 54.9% as compared to viruses (22.6%), parasites (19.4%) and fungi (3.1%) (Dhar et al., 2014; Kennedy et al., 2016). Fish diseases are categorized into two groups: diseases caused by indigenous pathogens, which are native to the fish environment; and diseases caused by foreign pathogens that are not normally found within the fish environment. Control of diseases caused by indigenous pathogens is attempted through good management practices and the use of approved drugs and/or vaccines. Such good management practices would include: maintaining good water quality, proper nutrition by ensuring good feed quality, good hygiene, and keeping the environment clean by following appropriate disinfection and sanitation protocols in order to minimize exposure to infectious agents (Defoirdt et al., 2011; Yanong & Francis-floyd, 2013). Control of diseases caused by foreign pathogens would include quarantine of new fish before being introduced into an existing group or population of fish and prevention of stress to fish by minimizing handling of fish and overstocking. Antibiotics, probiotics and vaccines have been on the forefront of bacterial disease prevention.

1.5.1 Antibiotics

Antibiotics have been used in aquaculture practices for many years and have been very successful in eliminating infectious diseases. Antibiotics such as penicillin, cephalosporin, rifampicin, diaminopyrimidines and bacitracin are the most effective antibiotics for treatment of Streptococcosis. In cultured rainbow trout, erythromycin administered orally with food is regarded as the most effective treatment (Kitao et al., 1987). Erythromycin, oxytetracycline, amoxicillin, doxycycline, lincomycin, tobicillin and penicillin are some of the antibiotics that have been used for the treatment of lactococcosis in rainbow trout. In Japan, oxytetracycline,

lincomycin and penicillin are reportedly used regularly, but have been shown to be relatively ineffective for treatment. Conversely, tobicillin, a more stable ester derivative of penicillin G, has been demonstrated to be very effective for the treatment of lactococcosis in rainbow trout. Table 1.2 gives a list of drugs that are approved for treatment of diseases in trout and other salmonid fish in South Africa. Other drugs that are not yet approved for use in the South African aquaculture industry include chloramphenicol, dimetridazole, and metronidazole (DAFF D: SAM, 2013).

Table1.2: List of drugs approved for use in South African aquaculture by the Department of Agriculture, Forestry and Fisheries, particularly for treatment of trout and other salmonoid fish in South Africa.

Drug	**Product name**	**Target**
florfenicol	Aquaflor Nuflor	freshwater reared salmonids
hydrogen peroxide	35% Perox- Aid	freshwater reared salmonids
formalin	Paracide-F	trout, salmon
sulfadimethoxine and ormetoprim	Romet 30	salmonids
oxytetracycline hydrocloride	oxytetracycline HCl-soluble powder –343 Terramycin-343 Tetroxy Aquatic- soluble powder	finfish fry
oxytetracycline dihydrate	Terramycin 200	freshwater reared salmonids and *O. mykiss*.

Infectious diseases, however, continue to cause problems and have been a major hazard to the aquaculture industry globally due to the development of bacterial resistance to antibiotics. Resistance to antibiotics has become a challenge to all aspects of chemotherapy (Sengupta & Chattopadhyay, 2012). As such, there has been an overwhelming concern over the use of antibiotics, particularly in aquaculture (Defoirdt et al., 2011). This is due to overuse or improper use of antibiotics in aquaculture systems, antibiotics included in and administered in feed to fish or added directly to the water system. These procedures result in selective pressure within the exposed environments that will affect a wide variety of bacteria and provide changes that will enhance transmission of resistant organisms, spread of

antimicrobial resistance genes and the accumulation of antimicrobial residues, eventually leading to more resistant and therefore virulent bacteria (Romero et al., 2012). A major problem that enhances this change is that bacterial species can survive unfavorable conditions or environmental changes, which enhances selective mutations that improve their fitness in the new conditions. Furthermore, bacteria take advantage of mobile genetic elements, such as plasmids and transposable elements that frequently harbor antimicrobial resistant and virulence genes. With these elements, bacteria can access a large pool of genes that move from one bacterial cell to another and can spread through bacterial populations (Romero et al., 2012; Defoirdt et al., 2011).

Diseases caused by antibiotic-resistant bacteria are difficult to treat, and there are very few new antibiotics being developed currently. For instance, in South Africa, Huchzermeyer & Henton (2011) listed descriptive factors that predispose rainbow trout to *Streptococcus* and how overuse of oxytetracycline led to the development of resistance towards oxytetracycline, inevitably making oxytetracycline ineffective for treating Streptococcosis causing bacteria. Such examples highlight the need for alternative antimicrobial compounds, to enhance protection, or alternative treatment strategies to improve general health and resistance to disease and improve the long term protection of aquatic animals (Defoirdt et al., 2011; Pridgeon, 2012).

1.5.2 Vaccination and vaccine types

Given the disadvantages of the use of antibiotics, vaccination has become important and also the most cost-effective disease management strategy used for controlling diseases in aquaculture. The major objective of vaccination is to induce long-term protection against a specific disease or disease agent (Muktar & Tesfaye, 2016). This protection is achieved by developing a vaccine that would present an inactivated structure of a pathogen to stimulate memory components of the host's specific immune system. A vaccine also achieves its objective by targeting a specific pathogen or specific structures of a pathogen. Therefore, before vaccine development can be initiated, various important aspects need be considered, such as the type of vaccine to be developed, its composition, possible mode of action, method of administration and most importantly its efficacy to achieve long term protection.

1.5.2.1. Types of vaccines

Different types of vaccines have been generated against bacteria responsible for streptococcosis and lactococcosis: live attenuated vaccines, killed inactivated vaccines, subunit vaccines and DNA vaccines.

1.5.2.1.1. Live attenuated vaccines

These vaccines are non-pathogenic or non-virulent strains that have the ability to stimulate the immune system without causing disease symptoms. They are composed of live attenuated microorganisms (bacteria, viruses) that have been grown in culture and no longer have the ability to cause significant disease. These vaccines have many advantages in aquaculture, including the ability to be administered in a single dose and the resulting immunity is long-lived. Immunization can result in both cellular and humoral immunity, resulting in good efficacy (Detmer & Glenting 2006). However, live attenuated vaccines have problems regarding their stability and safety. They are temperature sensitive, they require cold chain transport and storage and they have a relatively short shelf-life. Some of the disadvantages of using live attenuated vaccines include reversion to virulence and risk of spreading to non-target animals including wild fish populations (Muktar & Tesfaye, 2016). Although commonly used as human and animal vaccines, live attenuated vaccines are not commonly available in aquaculture. Ooyama et al. (2002) showed that a live attenuated *L. garvieae* strain lacking a virulence associated capsule on its cell surface can confer long-lasting protection to yellowtail (*Seriola quinqueradiata*). Buchanan et al. (2005) suggested the use of a live attenuated mutant *S. iniae* that lacks the virulence factor phosphoglucomutase to stimulate a protective immune response against Streptococcosis in rainbow trout.

1.5.2.1.2. Killed or inactivated whole organism vaccines

These vaccines are heat or chemically inactivated pathogens. These vaccines are known to elicit a potent humoral immune response but do not elicit a cellular response. Booster injections are almost always required. Inactivated whole organism vaccines against bacteria are composed of the entire cell content and therefore may contain toxins that can lead to local inflammation at the injection site as well as other side effects. Safety concerns associated with killed vaccines include the possibility of incomplete inactivation, variable potency and contamination and adverse reactions, such as local inflammation and seizures as caused by the pertussis vaccine in human. In spite of this, they are often used in aquaculture. Formalin-

killed *S. iniae* injected via the intra-peritoneal cavity showed specific antibody production and protection for over four months under both laboratory and field conditions (Kusuda and Salati, 1999). Sakai et al. (1987) demonstrated the efficacy of formalin-killed *S. iniae* in reducing infection rate in rainbow trout when injected via the intra-peritoneal (IP) or administered by immersion. Formalin killed *L. garvieae* have been used in a number of studies in rainbow trout. In a study by Romalde & Toranzo (2002), a *L. garvieae* oil adjuvanted bacterin, injected intraperitoneally in rainbow trout, provided low levels of protection for only 2-3 months, which was not long enough to provide protection for a following warm season when the majority of lactococcosis outbreaks occurred (Bercovier et al., 1997). For treatment of Weisselliosis, an oil-adjuvant vaccine and whole-cell inactivated bacterin were generated using a *W. ceti* strains that had previously been isolated from infected fish in Brazil and have been tested for efficacy in rainbow trout (Costa et al., 2014). The oil-adjuvanted vaccine was found to be the most effective vaccine, protecting rainbow trout against *W. ceti* infections at a relative percentage survival (RPS) of 92%. It has been observed that killed non-adjuvinated lactococcal and streptococcal vaccines provide protection for a limited period of time. However, increased protection time is usually observed when oil-adjuvants are added to the aqueous vaccine. The disadvantage to using adjuvanted vaccines is that they have been reported to hinder fish growth (Midtlyng and Lillehaug, 1998).

1.5.2.1.3. Protein subunit vaccines

Subunit vaccines are vaccines where only a specific protein antigen of a pathogen is used (Alpar, Papanicolaou & Bramwell 2005). Subunit vaccines are produced rapidly in cultured microorganisms, such as *Escherichia coli*, at lower cost (Alpar, Papanicolaou & Bramwell 2005). The disadvantage associated with this type of vaccine is low immunogenicity. A few studies have shown the protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to be a good candidate for vaccine development based on its ability to induce an immune response by production of protective antibodies. Tsai et al. (2013) studied a GAPDH isolated from an *L. garvieae* isolate which was shown to be a good candidate for vaccine development against lactococcosis in tilapia. A study by Zhang et al. (2017) also showed that the same GAPDH protein isolated from *S. agalactiae* could be used as a promising vaccine candidate in Nile tilapia. Another study by Trung Cao et al. (2014) also showed how effective an adjuvinated recombinant GAPDH (rGAPDH) vaccine, isolated from *E. ictaluri*, was against *E. tarda* infections in tilapia (*O. niloticus*).

1.5.2.1.4. DNA vaccines

A DNA vaccine is a DNA plasmid containing a pathogen antigen encoding gene. Upon delivery to eukaryotic cells, this leads to expression of the antigen encoding gene i.e. production of the antigenic protein. This elicits an immune response in the immunized animal and results in protection against the pathogen (Lewis & Babiuk 1999). DNA vaccines have several advantages over conventional vaccines in that only the antigen of interest is expressed and both antibody-mediated and cell-mediated immune responses are induced (Muktar & Tesfaye, 2016). There are, however, concerns associated with DNA vaccines, such as germ-line alterations and the induction of immune tolerance and autoimmunity (Alpar 2005). For instance, in a study by Ture & Altinok (2016), the existence of putative virulence genes in *L. garvieae* isolates from clinical rainbow trout from Turkey, France, Iran, Italy, Spain and yellowtail (*Seriola quinqueradiata*) from Japan were studied. The presence of several genes such as a capsule gene cluster (CGC), hemolysins, NADH oxidase, phosphogluco-mutase, adhesin, adhesin clusters, adhesin Pav, adhesin PsaA, enolase and superoxide dismutase LPxTG-(Leu-Pro-any-Thr-Gly) containing surface proteins, were studied as vaccine candidates. In spite of the overwhelming evidence about the capsule being the prime virulence feature used by pathogenic *L. garvieae*, Ture & Altinok (2016), concluded that a capsule is not essential for virulence of *L. garvieae* and it might be a combination of different genes that are responsible for the virulence of *L. garvieae*. Despite these concerns, numerous advantages of DNA vaccines make them an excellent approach for vaccine design (Alpar et 2005).

1.5.2.2. Vaccine efficacy and effectiveness

Vaccine efficacy and effectiveness for long term protection against bacterial diseases is one aspect of research that continues to challenge the practice of vaccination. There are several factors that influence vaccine efficacy: vaccine factors, host factors, administration factors, environmental factors and human factors. Vaccine factors would include: (1) the type of strain selected and quantity of the vaccine to ensure adequate coverage is obtained; (2) proper storage, for instance live and inactivated vaccines are fragile products and therefore preservation quality is of great importance (such as the expiry date must never be exceeded to ensure they remain viable) (Pritchard, 1965). The health status of the host should be monitored at all times before and after vaccination. One major host factor is failure to develop a protective immune response after vaccination, inevitably making the host

susceptible to infection. Environmental factors such as water temperature and stress during the immunization process will determine how quickly immunity will develop. Other factors such as crowding, handling and transport, can result in immune suppression and be a limiting factor for vaccine efficacy (Muktar & Tesfaye, 2016; Wali & Masood-ul-hassan, 2016). Human factors may not be fully considered to play an important role in vaccine efficacy but are important factors. Proper training and proper use of equipment by vaccinators is a requirement to ensuring effective administration of the vaccine (Pritchard, 1965).

1.5.2.3. Vaccine delivery strategies

The route by which a vaccine is administered can affect the outcome of vaccination. There are three main methods of administering vaccines to fish: Immersion by dip or bath, injection by intraperitoneal (IP) or intramuscular (IM) and oral vaccination (feed). These methods have different advantages and disadvantages as listed in Table 1.3 (page 22), with respect to the level of protection, side effects, practicality and cost-efficiency.

1.5.2.3.1 Immersion (dip or bath)

It is widely accepted that only the immersion and injection routes give enough protection to be used as the primary route of fish immunization in commercial production (Muktar & Tesfaye, 2016). Vaccination by immersion allows a large number of fish to be vaccinated in a short period of time and fish are only exposed to moderate stress. This is done (bath) by lowering the water volume and adding a known quantity of vaccine for a specific period of time, or by (dip) dipping a certain number of fish in a tank with a determined quantity of vaccine. One disadvantage is the quantity of vaccine needed to effectively immunize the fish, as it was found that a large quantity of vaccine is generally needed. However, high efficacy was observed when using live attenuated vaccines. For example, in a study by Pridgeon & Klesius (2011), a live attenuated *S. iniae* vaccine ISNO provided 88% protection to tilapia against a virulent *S. iniae* when administered by bath immersion.

1.5.2.3.2 Injection (intraperitoneal or intramuscular)

Comparisons of IP injection and bath immersion procedures for the same vaccine often demonstrate that IP injection offers better protection than bath immersion Pridgeon & Klesius (2011). In a study by Evans et al. (2004), a formalin-killed bacterin vaccine of *S. galactiae* (ARS-KU-MU-11B) provided 80% protection by IP injection to 30 g tilapia. However,

Table 1.3: Routes of vaccine administration used in aquaculture (Evensen, 2009).

Route of administration	**Type of formulation/delivery method**	**Advantages**	**Disadvantages**
Injection	○ Oil based (water in oil, oil in water) ○ Liposomes (experimental)	○ Most potent with little waste of vaccine ○ Allows the use of adjuvants ○ Cost effective method for high value species ○ Mass vaccination is possible	○ Stressful ○ Impractical for fish < 15 g ○ Labor intensive ○ Injection-site reactions ○ Immune response (level of protection)
Immersion (inactivated and live vaccines)	○ Used to a limited extent (mainly in marine fish species) ○ Live attenuated vaccines ○ Vector vaccines	○ Large scale application ○ Moderate stress to the fish ○ Easy - allows mass vaccination of immune-competent fish ○ High efficacy using live attenuated vaccines	○ Large amount of vaccine is needed ○ Low efficacy for inactivated vaccines ○ Inferior to injection routes in terms of efficacy ○ Cost prohibitive for large fish
Oral delivery	○ Top dressing ○ Formulation in polylactide-co-glycolide (PLG)	○ Imposes no stress to the fish ○ Moderate cost ○ All fish sizes can be vaccinated when immune-competent ○ Usually safe-primes mucosal immunity (external surfaces)	○ Usually low efficacy ○ Can be cost prohibitive for larger fish

immunization by bath immersion of the same sized tilapia (30 g) was less effective as compared to IP vaccination. Similarly, in another study by Soltani et al. (2010) on rainbow trout, an IP injection of a formalin-killed bacterin vaccine of *S. iniae* offered 74-100% protection, while immersion and oral vaccination offered 30-45% and 9-29% protection, respectively. Sakai et al. (1987) showed that a formalin killed bacterin vaccine of *Streptococcus* species offered protection to rainbow trout against virulent *Streptococcus* species through both IP injection and bath immersion with relative percent survival (RPS) of ≥70%. In another study by Klesius et al. (2000), the IM injection route was found to be less

efficient than the intraperitoneal injection route in some cases. For example, when a formalin-killed *S. iniae* ARS-10 vaccine was administered to tilapia by intraperitoneal injection, the RPS was 45.6% after fish were challenged with the homologous isolate *S. iniae* ARS-10. However, the RPS was only 17.7% when the same vaccine was administered by intramuscular injection.

Similarly, the same vaccine offered 93.7% protection to tilapia against challenge by a heterologous isolate *S. iniae* ARS-60 through intraperitoneal injection, whereas the same vaccine only offered 59.5% protection against the heterologous isolate ARS-60 when administered by intramuscular injection (Klesius et al., 2000; Gudding et al., 2014). When adjuvanted with Aquamun, a formalin–killed *L. garvieae* vaccine was reported to offer 92% protection in rainbow trout at 3 months post vaccination (Ravelo et al., 2006).

1.5.2.3.3 Oral delivery

Vaccines incorporated into feed are the most suitable means of mass vaccination of larger animals. However, the disadvantage can be poor potency due to uncertainty of dosage, where some fish will feed better than others, giving more potential for an immune response to those that consume more. Also, how viable and intact the feed (which carries the vaccine) remains in water plays an important role in the delivery to the host. The heat sensitivity of the antigen has to be considered and protected by coating an agent to either prevent leaching of the antigen from the pellets or to prevent breakdown of the antigen in the acidic environment of the stomach (Fig. 1.4) (Adams, 2016).

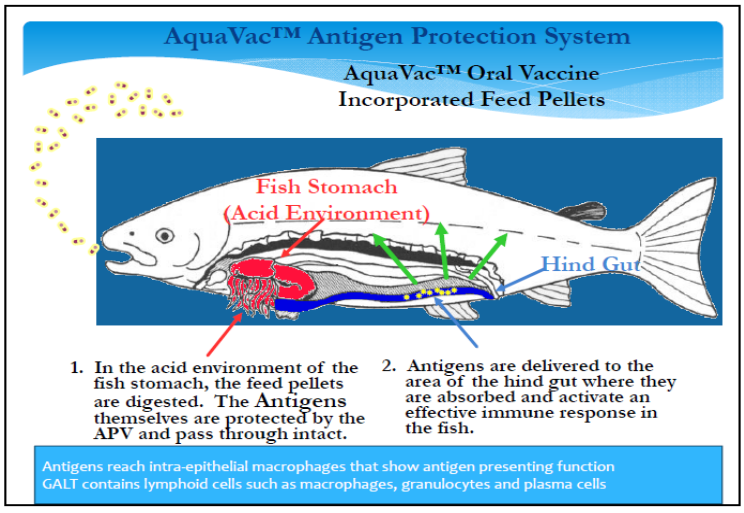

Figure 1.4: Figure representing the route of feed coated with vaccine (Adams, 2016).

Oral vaccination through feeding of vaccine is also used in aquaculture. This type of vaccination imposes no stress to the fish, and a large number of fish of different sizes can be vaccinated in a short period of time. In a study by Iida et al. (1982), a formalin-killed bacterin vaccine of *Streptococcus* species was evaluated in cultured yellow tail via three vaccination procedures (IP injection, oral feeding and hyperosmotic infiltration). The IP injection was found to be the most effective, followed by hyperosmotic infiltration and oral feeding. Kawai & Hatamoto, (1999) evaluated the use of oral vaccination with encapsulated and non-encapsulated antigens as an alternative immunization procedure against trout lactococcosis. Their results indicated that the algenate-encapsulated vaccines offered the best protective rates of oral immunization with a RPS of 50%. However, when injected, the algenate-encapsulated vaccine was not as effective and it was advised that this injection route should not be considered as the primary immunization method. When an aqueous-based *L. garvieae* bacterin vaccine was used on trout by IP injection as a primary immunization method, followed by a booster oral feeding of encapsulated vaccine, the RPS was increased to 87% (Kawai & Hatamoto, 1999).

1.5.2.4 Concluding remarks

The different routes of vaccine administration used in aquaculture, as discussed in the previous sections, are summarized in Table 1.3. Given the progress made so far with vaccine development for fish species, there are still major limitations such as costs and administration problems. Alternative vaccines such as DNA vaccines, recombinant subunit vaccine and vector-vehicle vaccine, subunit vaccines, polyvalent and monovalent vaccines and improved adjuvants are currently being explored for several fish diseases, but are highly dependent on biotechnology. Progress can be achieved by collaboration between researchers (immunologists, microbiologists and vaccinologists) which can open up opportunities for further research to new forms of highly efficient vaccines with ease of production, immunogenicity, safety, and multivalency in a single vaccine dose (Muktar & Tesfaye, 2016; Lee et al., 2012a).

1.6 Research aims and objectives

Since the first outbreak of Streptococcosis in South Africa during late-1970s, several outbreaks caused by Gram-positive bacteria have occurred over subsequent years, some of which have hampered the development of the trout industry in both South Africa and

Lesotho. Several Gram-positive bacteria have been isolated and characterized presumptively, but not at a molecular level and not with enough resolution to make a definitive diagnosis. In the current study, a total of 55 bacterial strains, that were isolated in the period between 2006 and 2012 from infected rainbow trout farmed in Lesotho and Mpumalanga Province of South Africa, were selected for characterization at a molecular level. A few of these isolates were previously used for vaccine development but were only effective for one or two seasons, highlighting the need to properly characterize the bacterial strains that had been isolated from diseased fish on rainbow trout farms in South Africa and neighboring Lesotho.

For these reasons, the objectives of this study were to:

1. Investigate the genetic diversity of the bacterial strains that had been isolated from diseased rainbow trout, by means of sequencing of the 16S rRNA gene followed by phylogenetic analysis;
2. Investigate differences between these bacterial isolates on the bases of phenotype, physiology, biochemistry, carbohydrate metabolism and antimicrobial susceptibility; and
3. Investigate the different serotypes that exist within this group of bacteria with rabbit produced polyclonal antibodies using an enzyme-linked immunosorbent assay (ELISA) with the subsequent objective of identifying bacterial strains suitable for vaccine development.

Accordingly, this thesis is structured as follows: Chapter 2 provides a detailed description of the different methodologies adopted for assessment of phenotype, physiology and molecular characterization of bacterial isolates. Chapter 3 describes the serological investigation performed with a developed enzyme-linked immunosorbent assay (ELISA). Chapter 4 contains the conclusions drawn from the study, highlights of the study, gaps that still need to be addressed and future plans that will contribute towards the development of vaccines against the pathogenic bacteria that occur in South Africa and Lesotho identified in this study.

CHAPTER 2: GENOTYPIC, BIOCHEMICAL AND PHENOTYPIC ANALYSIS

2.1 INTRODUCTION

For many years, morphological, phenotypic and biochemical characterization methods were the only methods available for the identification of bacteria (Buller, 2014). Primary methods such as Gram staining, which identifies bacterial cell structure; the oxidase test, to confirm the use or nonuse of oxygen for the electron transport chain; the catalase test, which also confirms bacteria's ability to live in an oxygenated environment; would give basic information necessary to streamline a species identity. Biochemical identification methods such as the Biomerieux produced Analytical Profile Index (API) systems are very useful in identifying bacteria at a species level [API 50CH, API 20 Strep, API Rapid ID 32 Strep, API 20E/NE and API- Staph] (Buller, 2014). Regardless of the amount of time it takes to use such methods, they are still an important diagnostic tool as they rely on fermentation, hydrolysis or carbon source utilization of a specific substrate of metabolism.

However, the use of these conventional methods to identify bacteria at a species level has been met with difficulties due to either intraspecies or interspecies phenotype diversity (Buller, 2014). Molecular identification methods, on the other hand, have become powerful alternatives to conventional methods of bacterial differentiation. Polymerase Chain Reaction (PCR) is one easy-to-use molecular technique that has become increasingly useful in detecting and identifying different bacterial pathogens. The sequencing of the 16S rRNA gene has been very useful in providing information about the evolutionary diversity of the bacterial community. Several studies have demonstrated or emphasized the need to use PCR as a complementary and supplementary test for definitive identification of bacteria isolated from clinical samples, particularly those related to the disease Streptococcosis (Karsidani et al., 2010; Pourgholan et al., 2011; Kia & Mehrabi, 2013; Buller, 2014). Given the wide distribution of bacterial species, molecular characterization therefore becomes crucial for epidemiological purposes, obligatory for identifying transmission pathways and genetic relatedness among strains (Foschino et al., 2008). However, for some genera, the 16S rRNA gene is so similar or conserved between species that it offers little value for diagnostic identification, unless used in conjunction with other identification methods (Buller, 2014). A good example for this would be in the identification of *V. salmoninarum*, which shares 92-94 % similarity with the 16S rRNA gene sequence of *Carnobacterium* and *Enterococcus*

species. It also shares 96% 16S rRNA gene sequence similarity with *V. fluvialis* (Wallbanks et al., 1990; Buller, 2014).

Gram-positive bacteria belonging to the genus *Streptococcus* have been the cause of recurring disease outbreaks on South African trout farms, as well as in neighbouring Lesotho Bekker et al. (2011). It is therefore crucial to consider both molecular based PCR and conventional methods to gain a broader understanding and knowledge of the identity and differences shared between the bacterial isolates analyzed in this study. PCR and sequencing of the 16S rRNA gene of bacteria isolated from diseased farmed trout over the last decade was conducted to identify and characterize bacteria at a molecular level. To get further understanding of growth preferences of these bacterial species, biochemical and physiological studies were performed, where these bacteria were exposed to different growth conditions such as different ranges of pH, temperature and salinity. This information is necessary for providing insight on intra- and/or inter-species differences among bacteria. The results of this study will help inform the selection of appropriate bacteria for the development of a vaccine that will cover all strains and/or isotypes of bacteria responsible for Streptococcosis.

2.2 MATERIALS AND METHODS

2.2.1 Maintenance of bacterial strains and general growth conditions

During the period of 2006 to 2012, a total of 55 bacterial strains were obtained by Dr Anna Mouton and Dr David Huchzermeyer from trout farms in Lesotho and farms in Mpumalanga, KwaZulu Natal, Limpopo provinces of South Africa (the localities of which are shown in colour in Fig. 2.1) that reported high mortalities of fish with clinical symptoms of Streptococcosis. Bacteriological samples were taken aseptically from kidney and liver tissues of diseased trout and inoculated onto blood agar plates (Difco) for the isolation and preparation of pure cultures. All pure cultures were freeze dried and stored at Design Biologix CC in Pretoria, South Africa until they were used for this study. Freeze dried cultures were then cultured on Trypicase soy broth after which they were cultured by spread plating on blood agar plates by Dr Anna Mouton, Amanzi Biosecurity, Hermanus. At the Aquaculture Research Aquarium in Cape Town, the bacteria were received on blood agar plates and then subcultured aerobically on trypticase soy agar (TSA) (Biolab) plates at 24 ºC for 48 hrs. For long term preservation, bacteria were stored in nutrient broth (Biolab) supplemented with 20% (v/v) glycerol at -80 ºC.

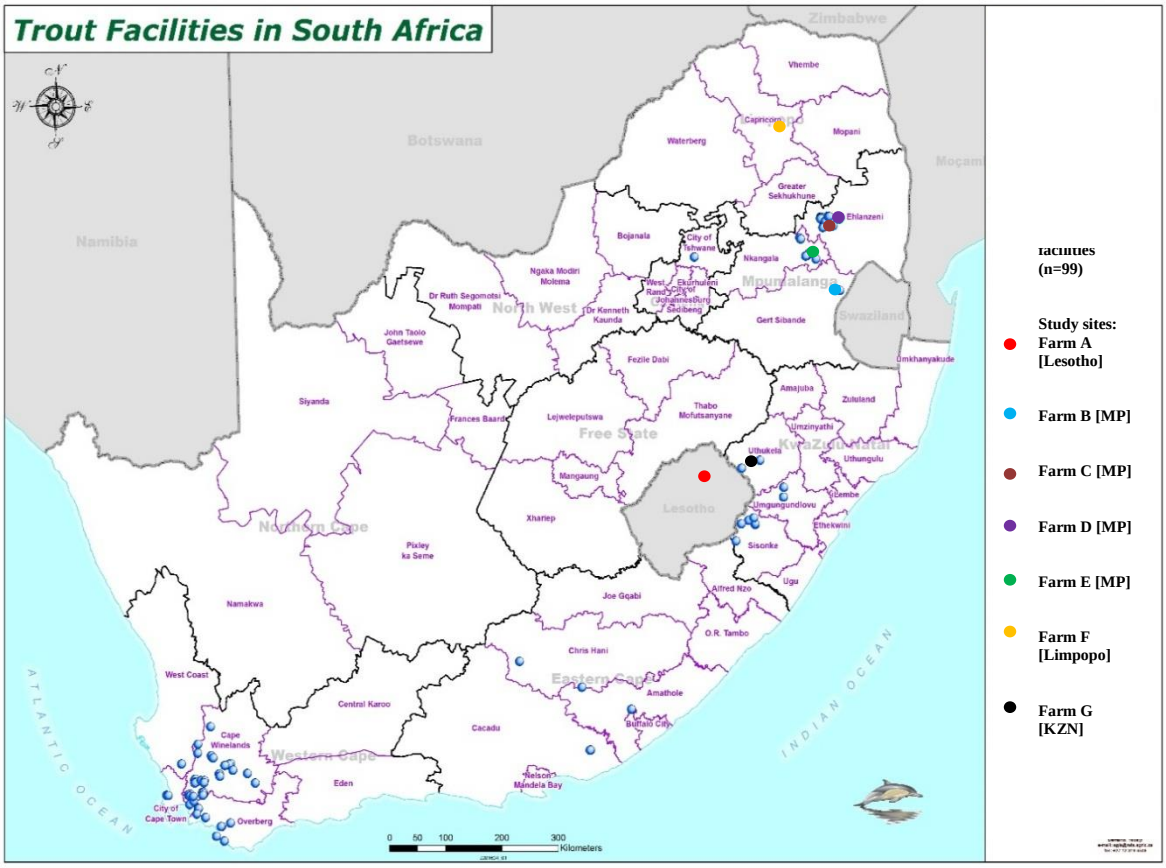

Figure 2.1: Map showing trout facilities in S.A and sites from where isolates used in this study were collected. Number of isolates from each farm: **Farm A= 22**, **Farm B= 16**, **Farm C= 8**, **Farm D= 5**, **Farm E= 1**, **Farm F= 2** and **Farm G= 1**.

2.2.2 Molecular characterization

2.2.2.1 DNA extraction and polymerase chain reaction protocol

Genomic DNA was extracted from each bacterial culture using a simple heat lysis method. Briefly, cells from a single colony of each bacterium were suspended in 25 µl Tris-EDTA (TE, pH 7.6) buffer and lysed at 100 ºC for 2 min. The lysed cell suspension was cooled on ice before being centrifuged at 10,000 × g for 2 min. The resulting supernatants (20 µl), containing genomic DNA, were transferred to a new sterile microcentrifuge tube and stored at -20 ºC until needed. An aliquot of 2 µl of each DNA stock was used for Polymerase Chain Reaction (PCR) analysis.

The 16S F-fD1 and 16SR-Rp2 primers (Table 2.1) were used for PCR to amplify the 16S rRNA gene of each isolate. The PCR reaction mixtures (25 µl) were prepared using 2 µl genomic DNA; 12.5 µl of 2 × KapaTaq ReadyMix (containing 0.05 U/µl DNA Polymerase (1.25 U per 25 µl), supplemented with 1.5 mM $MgCl_2$ and 0.4 mM of each dNTP); 0.5 µl of each primer; and 9.5 µl of PCR grade water. Amplification was conducted using a Labnet Multigene^{TM} thermal cycler and consisted of an initial denaturation step at 96 °C for 3 min, followed by 40 cycles of DNA denaturation/melting at 96 °C for 45 sec, annealing at 57 ºC for 30 sec and elongation at 72 °C for 1 min, with a final elongation step at 72 °C for 5 min. The amplified PCR products were analysed by agarose (0.8 %) gel electrophoresis to verify reaction specificity and fragment size (~1.5 kb fragment expected).

Table 2.1: Oligonucleotide primers used in this study (10 µm concentration each) (Barghouthi, 2011).

Primer	Direction	Nucleotide sequence (5'-3')	Fragment size
16S F-fD1	Forward	AGAGTTTGATCCTGGCTCAG	1.5 kb
16SR-Rp2	Reverse	ACGGCTACCTTGTTACGACTT	
16S F3	Forward	GCCAGCAGCCGCGGTAATAC	1.0kb
16S R3	Reverse	CACGAGCTGACGACAICCATG	

2.2.2.2 Sequencing and phylogenetic analysis

PCR products were purified using a GFX microspin column kit according to the manufacturer's instructions. The DNA containing samples were eluted in 50 µl of Milli-Q water and stored at -20 °C. Sequencing was performed at Stellenbosch University Sequencing Unit. The purified 16S rRNA PCR products were sequenced using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) and ABI3730xl Genetic Analyzer (Applied Biosystems) according to the manufacturer's instructions. Primers F-fD1, R-Rp2 and F3 (0.8 µl of each; Table 2.1) were utilized for cycle sequencing. ChromasPro was used for editing chromatograms and assembling consensus sequences from three sequences that were obtained for each strain (F-fD1, R-Rp2 and F3). The consensus sequences (each approximately 1000-1200 bp in length) were saved in FASTA format and homology searches were carried out using the BLASTN algorithm (Altschul et al., 1989) provided by the Internet

service of the National Centre for Biotechnology Information (http://www.ncbi.blast.nlm.nih.gov/BLAST/).

BioEdit version 7.1.11.0 was used for sequence alignments on the newly sequenced isolates and of selected outgroups as indicated in Appendix B, Table B.2 . Multiple sequence alignments (MSA) were performed using ClustalW and the matrices of aligned sequences were trimmed at the 5' and 3' ends in order to exclude missing characters. The resulting alignments were exported for construction of phylogenetic trees using parsimony analysis, which were conducted using Phylogenetics Analysis Using Parsimony (PAUP) 4.0b10 software, the Macintosh version (Swofford, 2002). A heuristic search was used to establish the shortest possible trees from the data matrices. The search criteria included the use of 1,000 addition sequence replicates with tree bisection and reconnection (TBR) branch swapping and starting trees obtained via stepwise addition. With each repetition, only the shortest trees were kept and no more than 10 trees (MulTrees) with a tree score larger than or equal to five were saved, with all characters equally weighted. A strict consensus tree was computed from the shortest trees found in the heuristic search. A bootstrap analysis of 1,000 replicates using TBR branch swapping, MulTrees off, was performed to establish clade support. Branches with bootstrap values ≥ 75% were considered to be well supported, whereas values between 75% and 50% were considered as moderately supported. Values below 50% were considered weakly supported and not indicated on the phylograms. Trees were saved as PICT files (Apple Macintosh metafile/ graphics format) and also viewed on Windows Power point for editable version.

2.2.3 Physiology, phenotype and biochemical analysis

2.2.3.1 Phenotype

Differences in phenotypic characteristics of each bacterial isolate were assessed by examining the colony morphology, cell shape, Gram-reaction and motility.

2.2.3.1.1 Colony morphology

Colony morphology was assessed following growth on two media, TSA and Colombia blood agar (BA) plates containing 4% horse blood. Colony form (shape, color, size & texture), elevation (flat, raised, umbonate, convex or pulvinate) and margin (entire, undulate, lobate or filiform) were observed and recorded after 48 hrs of growth at 24 ºC.

2.2.3.1.2 Gram stain

The Gram reaction was tested following the general procedures of Wiley et al. (2008). Briefly, cells fixed on a microscope slide were stained with Crystal violet solution for 30 sec, rinsed gently with running water and air dried. Cells were then flooded with iodine solution (mordant) for 2 min, decolourized with alcohol for a few seconds, rinsed with water and air dried. Finally, cells were counterstained with Safranin for 1 min, rinsed with water, air dried and then examined under a light microscope equipped with a 100× oil immersion lens.

2.2.3.1.3 Motility

A total of 26 bacterial isolates were tested for motility: 14 strains from South Africa and 12 strains from Lesotho. The Hanging drop method was used for assessing motility as described by Smibert & Krieg (1994). Two other bacterial isolates were utilized as controls: *Vibrio anguillarum* as a positive control and *Escherichia coli* as a negative control.

2.2.3.2 **Biochemical analysis**

2.2.3.2.1 Oxidase test

All 55 isolates were tested for their ability to produce cytochrome oxidase, an enzyme that plays an important role in the electron transport system during aerobic respiration. Cells from a single colony of each isolate were smeared onto the paper zone of a diagnostic strip (Merck Microbiology Bactident Oxidase). After 1 min, the colour on the strip was recorded, with dark-blue to black indicating a positive reaction and no change in colour indicating a negative reaction. *V. anguillarum* was included as a positive control and *E. coli* as a negative control.

2.2.3.2.2 Catalase test

All isolates were examined for their ability to produce catalase, an enzyme that catalyses the decomposition of hydrogen peroxide (H_2O_2) to oxygen and water during aerobic respiration. Cells from a single colony were transferred using a sterile pipette tip onto a drop of 3% H_2O_2 placed on a glass microscope slide. The generation of bubbles after a few seconds indicated a positive test, whereas the absence of bubbles indicated a negative result. *Vibrio anguillarum* and *E. coli* were used as positive and negative controls, respectively.

2.2.3.2.3 Haemolytic activity

Bacterial haemolytic activity was assessed following growth on BA plates. Each bacterial isolate was initially grown on TSA at 24 °C for 48 hrs before being sub-cultured onto BA plates. Inoculated plates were incubated at 24 °C and the results recorded after 48 hrs of incubation. The presence of dark and/ or greenish coloration of the agar under a colony indicated beta-haemolysis (β-haemolysis); no change in colour represented a lack of haemolytic activity and was recorded as gamma-haemolysis (γ-haemolysis); and lastly, as alpha-haemolysis (α-haemolysis) where there was slight haemolytic activity.

2.2.3.2.4 Carbohydrate metabolism

Based on the results of the phylogenetic analysis obtained in this study, only 20 (due to the number of determinations in the kit) of the bacterial isolates were selected for assessment of carbohydrate metabolism using the Analytical Profile Index (API) 50CH system (BioMérieux, Inc.). Isolates were initially cultured on TSA directly from glycerol stocks. After 24 hrs of incubation at 24 ºC, a single colony of each strain was sub-cultured onto BA plates before using the API 50CH system. After 48 hrs of growth on BA plates, cells from each isolate were suspended in separate vials containing 6 ml API 50 CHL medium to achieve an absorbence value of 0.8±0.1 at $OD_{540,}$ as prescribed by the manufacture, with the exception of using 6 ml of medium instead of 10 ml provided by manufacturer. The API 50CH strips were inoculated with 100 µl of inoculum and sealed with mineral oil as prescribed by the manufacturer. They were incubated at 30 °C for 48 hrs. The biochemical profiles were recorded on the results sheet provided by the manufacturer and identified using the apiweb™ identification software (BioMérieux, Inc.).

2.2.3.3 Physiological analysis

The same 26 isolates previously used for assessment of carbohydrate metabolism were characterized further by assessing their growth at different temperatures, salinity and pH. The isolates were cultured on TSA plates from glycerol stocks and incubated at 24 °C for 48 hrs.

2.2.3.3.1 Growth at different temperatures

The growth of each isolate at four different temperatures (10, 30, 37 and 45 °C) was assessed after 24 hrs of incubation in TSB. A single colony from each TSA plate was then suspended in 5 ml TSB and grown for 24 hrs at 24 °C. Each 100 ml flask containing 15 ml of TSB broth was inoculated with an overnight culture of bacteria to achieve an initial optical

density OD_{600} of 0.1 and incubated at 24 °C on an orbital shaker set at 100 rpm. Triplicate assays were performed for each isolate at each respective temperature, and the OD was assessed at 6 hrs of incubation and again at 24 hrs of incubation for each particular temperature tested.

2.2.3.3.2 Growth at different NaCl concentrations

Tolerance to different NaCl concentrations was determined following growth in Luria broth (LB) medium supplemented with varying concentrations of NaCl (0, 3.5, 5 and 6.5 % w/v). LB was selected for use because TSB contains 5% NaCl and will not be useful for assessing growth in media containing 0% NaCl. The isolates were initially cultured on TSA plates (pH 7.2) and incubated at 24 ºC for 48 hrs. A single colony from each plate was then suspended in 5 ml TSB (pH 7.2) for 24 hrs before being transferred to 100 ml flasks containing 15 ml of LB supplemented with the varying concentrations of NaCl listed above. Each isolate was inoculated to achieve an initial OD_{600} of 0.1 and was incubated at 24 ºC on an orbital shaker set at 100 rpm. All assays were performed in triplicate and the OD_{600} recorded at 6 hrs and 24 hrs of growth.

2.2.3.3.3 Growth at different pH

Ability to grow at different pH values was assessed by adjusting the pH of the growth media to pH 4.5, 5, 7, 8 and 9.5. The isolates were initially cultured on TSA plates (pH 7.2) and incubated at 24 ºC for 48 hrs. A single colony from each plate was then suspended in 5 ml TSB and grown for 24 hrs at 24 °C. Cultures were inoculated in 100 ml flasks containing 15 ml TSB adjusted to pH 4.5, 5, 7, 8 and 9.5. Each broth was inoculated to achieve an initial OD_{600} of 0.1 and were incubated at 24 ºC on an orbital shaker set at 100 rpm. The absorbance readings were taken at 6 hrs and at 24 hrs of growth.

2.2.3.3.4 Statistical analysis

All statistical analyses were performed using Statistica 13, Dell Inc. 2015 (www.statsoft.com), using data recorded after 24 hrs of each test and included three replicates of each isolate. Repeated Measures ANOVA was run to assess and compare growth between the isolates at each different temperature, pH and salt concentration. The graphs were plotted using the Least Square Means (LSM) for all variables; variables being temperature (10, 24, 30, 37 and 45 °C), pH (4.5, 5, 7, 8 and 9.5) and salinity (0, 3.5, 5 and 6.5%). A descriptive analysis was run to view the means and standard errors (which were included on the bar

graphs) and all post-hoc analysis was conducted with Tukey Honest Significance Difference (HSD). Significance was assigned to P-values of <0.05 for all analysis.

2.2.4 **Antimicrobial susceptibility**

2.2.4.1 Bacterial culture preparation

The susceptibility or resistance patterns of 26 selected isolates to eleven antibiotic agents (Table 2.2) were assessed using the disc diffusion method as described by Kirby & Bauer, (1960). The bacterial strains were cultured on TSA plates and incubated at 24 °C. After 24 hrs of growth on TSA plates, 5 ml TSB cultures were prepared to obtain a final OD of McFarland standard 0.5. The optical density of each culture was read at OD_{600}, which equated to an absorbence value of between 0.08 to 0.10 (comparable to 1-2 × 10^8 cells/ml) (Biologicals, 2014; Marie, 2005).

2.2.4.2 The disc diffusion method

All the antibiotics utilized in this study are listed in Table 2.2 and were prepared according to the Clinical and Laboratory Standards Institute (CLSI, 2014; Raissy & Ansari, 2011). The antibiotic working stocks were stored at -20 °C until needed. Discs of 6 mm were prepared from Whatmann paper and sterilized by autoclaving at 121 °C for 15 min. For inoculation with each of the tested strains, a dry sterile swab was dipped in the bacterial culture, excess liquid pressed on the sides of the tube, and inoculated onto a TSA plate by spreading, covering the entire surface of the plate. The plates were allowed to dry for at least 2 hrs on the bench before the discs were applied. Plates were then incubated at 24 °C for 24 hrs and the results recorded as a measure of the diameter minus the disk in mm to determine the size of the inhibition zone.

Table 2.2: Antibiotics that were utilized for the analysis.

Mode of action	Antibiotic group	Members and dose	Range of reactivity (mm)		
			R	I	S
Cell wall synthesis inhibition	Penicillins	Penicillin G (10 µg)	≤11	=12-17	≥18
	Glycopeptides	Vancomycin (30 µg)	≤10	=11-15	≥16
	Cephalosporins	Cefotaxime (10 µg)	≤22	=23-25	≥26
Protein synthesis inhibition	Macrolides	Erythromycin (15 µg)	≤13	=14-17	≥18
	Aminoglycosides	Gentamicin (10 µg)	≤12	=13-14	≥15
		Streptomycin (10 µg)	≤11	=12-14	≥15
		Kanamycin (30 µg)	≤12	=13-15	≥16
	Tetracyclins	Oxytetracycline (30 µg)	≤13	=14-17	≥18
	Phenicols	Chloramphenicol (30 µg)	≤12	13-17	≥18
Nucleic acid inhibition	Quinolones and fluoroquinolones	Norfloxacin (10 µg)	≤12	13-16	≥17
Antimetabolites	Sulfonamides	Sulphamethoxazole (25 µg)	≤10	11-15	≥16

R= resistant, I= intermediate, S= susceptible. Doses were chosen based on the literature, see Appendix A Table A.2.

2.3 RESULTS AND DISCUSSION

Of the 55 bacterial isolates evaluated in this study, 21 bacteria originated from trout facilities in Lesotho and 34 from trout facilities in South Africa. From the PCR reactions that were performed, PCR products of approximately 1,500 base pairs (bp) were successfully obtained for each isolate and prepared for sequencing. Consensus sequences were determined and were deposited in the GenBank database (Table 2.9). A BLAST search of the GenBank database revealed that *Lactococcus* was the predominant genus isolated from diseased trout in both countries, with 50 out of the 55 isolates showing high sequence similarity (>98%) to organisms in the genus *Lactococcus*. Approximately 90% (n=49) of the isolates belonged to the same genospecies, *L. garvieae*, with only one isolate identified as a *Lactococcus* species with high sequence similarity to *L. lactis* (Fig. 2.2). Two of the isolates from South African trout showed high sequence similarity to *Weissella* species (Fig 2.15), whereas three of the isolates showed high sequence similarity to *Carnobacterium maltaromaticum* (Fig 2.10).

2.3.1 Lactococcus garvieae and *Lactococcus* species

L. garvieae is one of the most pathogenic species that has been reported most frequently from diseased freshwater fish worldwide and have caused major problems within the

aquaculture industry (Aguado-Urda et al., 2011; Miyauchi et al., 2012). The first lactococcosis outbreak in Rainbow trout was reported from Spain in 1988 (Ghittino & Prearo, 1992). Since then, *L. garvieae* has caused severe mortalities in the rainbow trout sector in many countries worldwide (Tanrikul & Gultepe, 2011). *L. garvieae* strains are said to be phenotypically homogeneous, regardless of their geographic location or the aquatic host from which they were isolated (Buller, 2014). This notion was supported by Ture & Boran (2015), who showed that *L. garvieae* strains isolated from diseased fish from different regions around Turkey (seventeen isolates), Italy (four isolates), Spain (six isolates) and France (one isolate) were biochemically similar to one another. Results obtained from the present study are in agreement with these previous findings, with strains from both regions (Lesotho and South Africa) exhibiting high similarity to each other both biochemically and phylogenetically. The phylogenetic analysis revealed that isolates from South Africa (n=30) and Lesotho (n=20) are closely related to *L. garvieae* strains that had previously been isolated from diseased rainbow trout, exhibiting clinical signs of lactococcosis in China, Japan and Taiwan. The *Lactococcus* sp. KM409665 from Lesotho formed a separate clade with *L. lactis* (Fig. 2.2). The phylogeny also shows that *L. garvieae* and *L. lactis* are closely related which is in agreement with their morphological and growth characteristics as they are Gram-positive, cocci, non-motile facultative aerobes and are phenotypically indistinguishable (Buller, 2014).

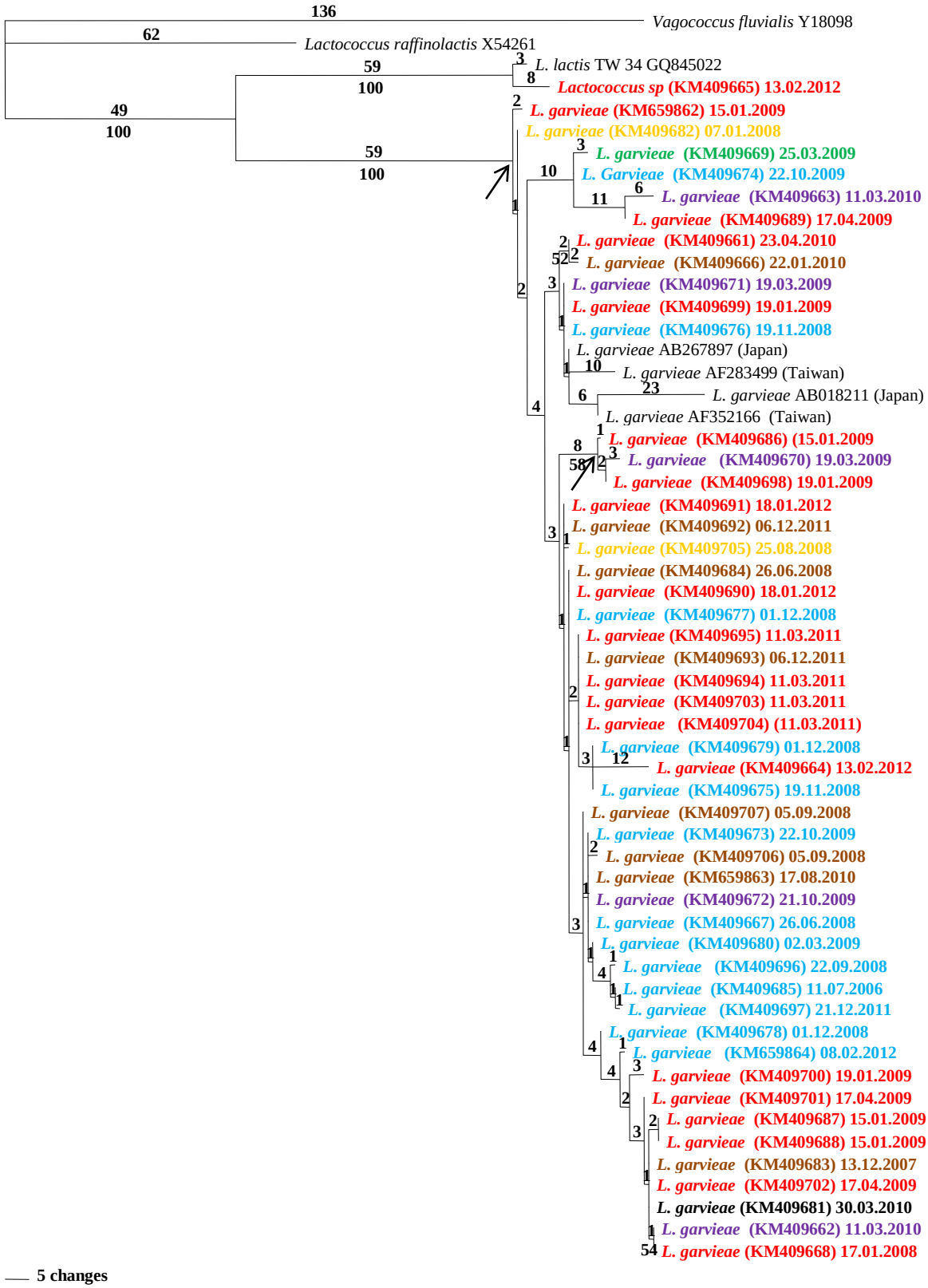

Figure 2.2: One of the shortest trees retrieved from the heuristic search using parsimony of *L. garvieae* 16S rRNA gene sequences. Results revealed 560 trees with a total length of 535. The alignment matrix consisted of 1226 (77%) constant, 168 parsimony informative and 197 parsimony uninformative characters. Branch lengths are shown above branches, bootstrap percentages shown below branches and branches that collapse in the strict consensus are indicated with an arrow. The colours relate to their origin (**Farm A**, **Farm B**, **Farm C**, **Farm D**, **Farm E**, **Farm F** and **Farm G**).

L. garvieae is known to be facultative anaerobic, Gram-positive, catalase and oxidase negative cocci. Of the 55 isolates evaluated, 50 were identified as Gram-positive cocci with circular, umbonate (margin) and undulate elevated whitish colonies of approximately 2 mm in diameter. Furthermore, all 50 colonies were catalase and oxidase negative. Differences in haemolytic activity were however noted among the *L. garvieae* isolates, with two types of haemolysis recorded: γ-haemolysis (n=28) and α-haemolysis (n=22) (Fig 2.3).

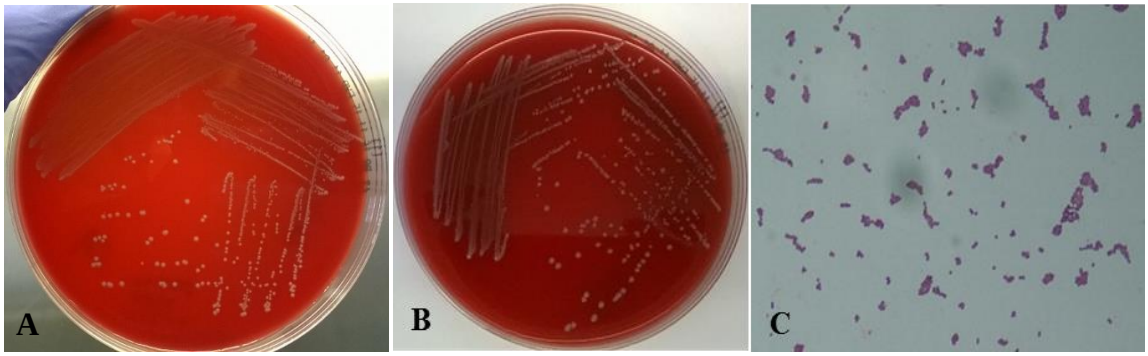

Figure 2. 3: Growth of *L. garvieae* on blood agar plates: A= γ-haemolysis (n=28) & B= α-haemolysis (n=22) and Gram-positive cocci (C).

L. garvieae is stated to grow between 10-40 ºC and strains may have weak to delayed growth at 45 ºC (Buller, 2014). From the present study, we demonstrated weak to no growth of *L. garvieae* strains at 10 ºC, even after 48 hrs of growth. Among the South African isolates (Fig. 2.4), isolate KM409663 was significantly different from the rest of the isolates in its growth at 10 °C, while isolates KM409673, KM409680, and KM409682 grew weakly at 10 ºC (significant to 0.05). None of the Lesotho isolates grew at 10 ºC by 24 hrs, with the exception of *Lactococcus* isolate KM409665. The latter isolate grew very well at 10 ºC, when compared to the other *L. garvieae* isolates from Lesotho that were tested in this study (Fig. 2.5). *Lactococcus* isolate KM409665 grew non-significantly better at 30 ºC than at 37 and 45 ºC (Fig. 2.5), a finding not too surprising since *L. lactis* isolates have been shown to grow best at temperatures between 4-40 ºC (Buller, 2014). Conversely, all of the *L. garvieae* isolates grew optimally at 37 ºC (Fig. 2.4 & 2.5), while growth at 45 ºC was highly variable for most isolates tested in this study. Most notably, isolate KM409685 failed to grow at 45 ºC ($p= 0.000201$) as compared to the rest of the South African isolates. This would imply that *Lactococcus* isolate KM409665 is in all likelihood *Lactococcus lactis*.

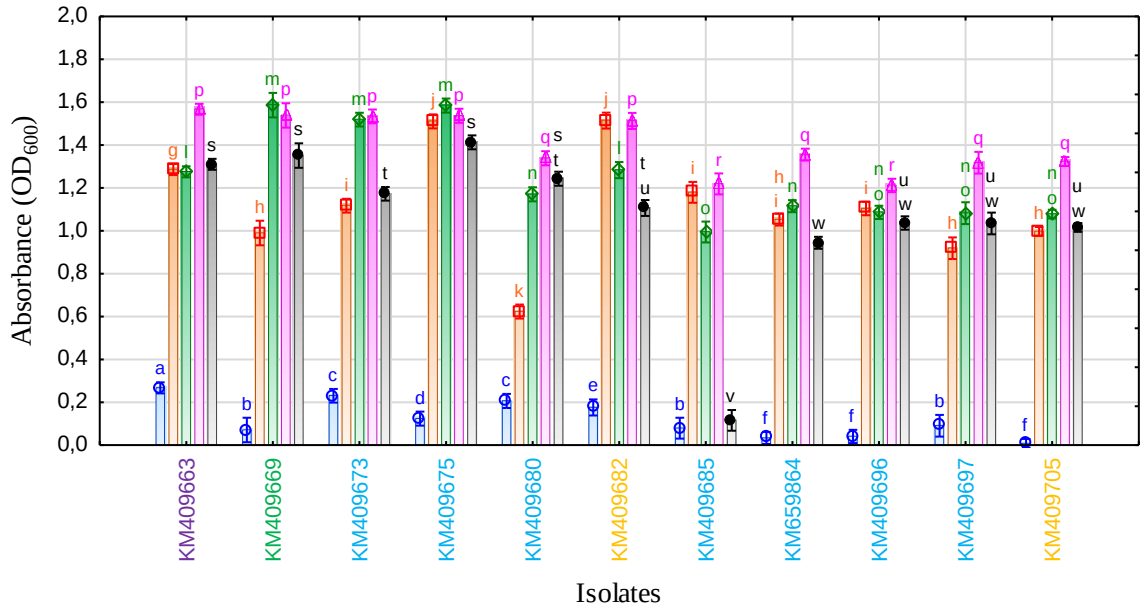

Figure 2.4: Graph showing mean (±SEM) growth of bacterial isolates from South African trout **(Farm B, Farm D, Farm E, Farm F)**, at different temperatures (**10 °C, 24 °C, 30 °C, 37 °C, 45 °C**). Different alphabetical letters denote a significant difference in growth of different isolates at a specific temperature.

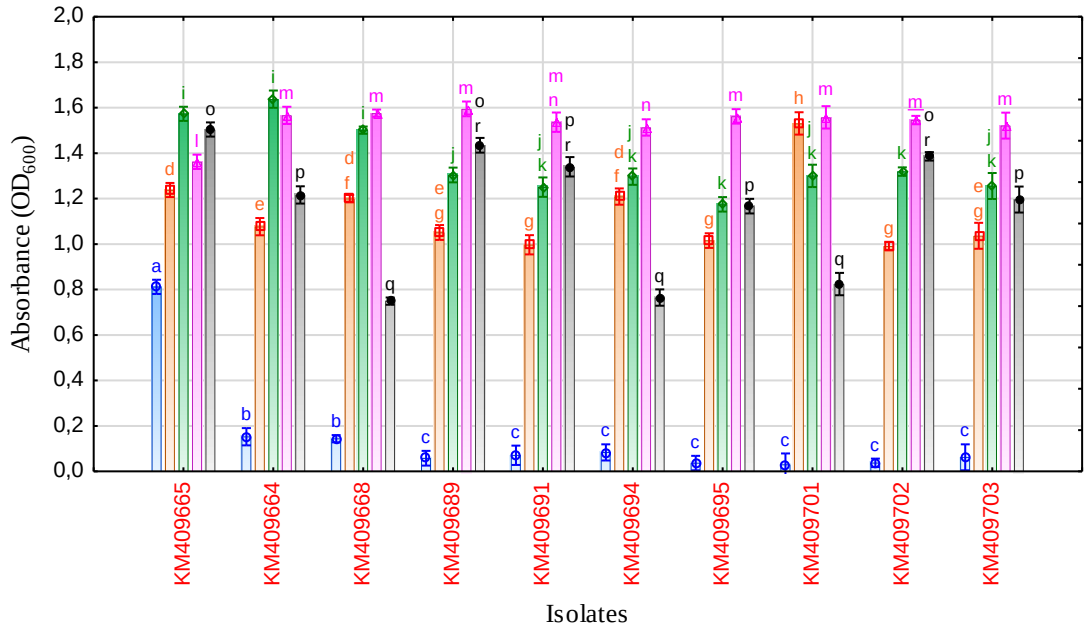

Figure 2.5: Graph showing mean (±SEM) growth of bacterial isolates from Lesotho (**Farm A**), at different temperatures (**10 °C, 24 °C, 30 °C, 37 °C, 45 °C**). Different alphabetical letters denote a significant difference in growth of different isolates at a specific temperature.

Based on the pH results obtained from this study (Fig. 2.6 & 2.7), weak growth was observed at pH 4.5 for all isolates. In particular, little to no growth was observed for isolates

KM409673, KM409680, KM409685 (farm B) and KM409682 (farm F). The remaining isolates showed weak growth, achieving absorbance values of between 0.2-0.4 nm. Among the Lesotho isolates, only three isolates (*Lactococcus* isolate KM409665, KM409664, and KM409689) displayed weak growth, with absorbance readings below 0.4 observed, whereas the remaining did not grow at all. All *L. garvieae* isolates grew best in media with a pH of between pH 7-9.5, with absorbance values of above 1.0 obtained in each case. Significant differences in growth were however observed for all *L. garvieae* isolates at the different pH values tested (Fig. 2.6 & 2.7).

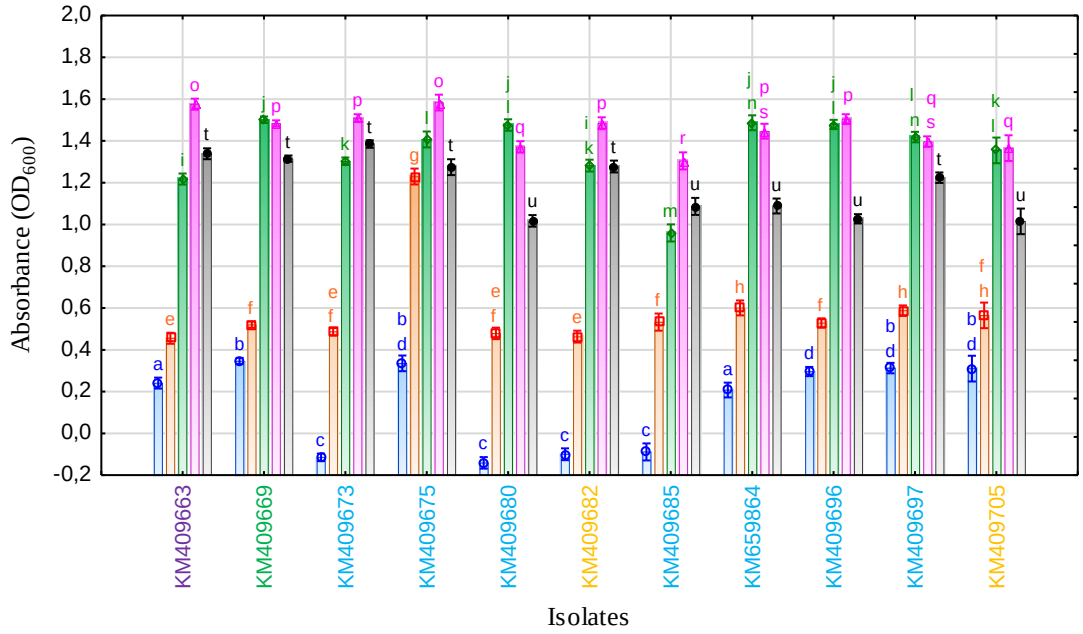

Figure 2.6: Graph showing mean (±SEM) growth of bacterial isolates from South African trout (**Farm B, Farm D, Farm E, Farm F**), grown at different pH values (**pH 4.5; pH 5; pH 7; pH 8; pH 9.5**). Different alphabetical letters denote a significant difference in growth of different isolates at a specific pH.

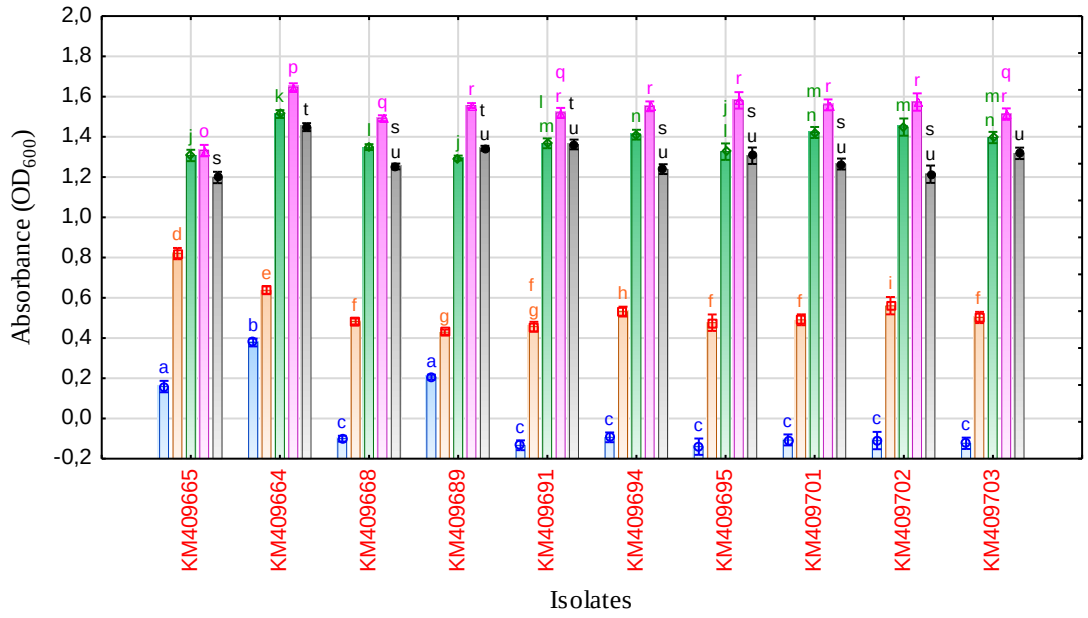

Figure 2.7: Graph showing mean (±SEM) growth of bacterial isolates from Lesotho (**Farm A**), grown at different pH values (**pH 4.5**; **pH 5**; **pH 7**; **pH 8**; **pH 9.5**). Different alphabetical letters denote a significant difference in growth of different isolates at a specific pH.

L. garvieae is reported to grow at NaCl concentrations ranging between 0-6%, with weak growth expected at NaCl concentrations of 6.5% and above (Buller, 2014). Data from this study showed that all isolates (from both South Africa and Lesotho) grew best in media with 0 % NaCl, with the exception of isolate KM409685, which did not grow with an $OD_{600} < 0.1$ (Fig. 2.8) (significant to 0.05). The second best recorded growth in all isolates was at 3.5% NaCl. Very weak growth occurred in media supplemented with 5% NaCl amongst all the South African isolates, with three isolates (KM409669, KM409685 and KM659864) not growing at all ($OD_{600} \leq 0$; Fig. 2.8). Similarly, with the Lesotho isolates weak growth was recorded in media supplemented with 5% NaCl from only two isolates (KM409691 & KM409701), while no growth occurred among the remaining isolates. *Lactococcus* isolate KM409665 grew best at 0% NaCl, with weak growth recorded at 3.5% NaCl and no growth recorded in media supplemented with 5 or 6.5% NaCl ($OD_{600} < 0.1$ (Fig. 2.9).

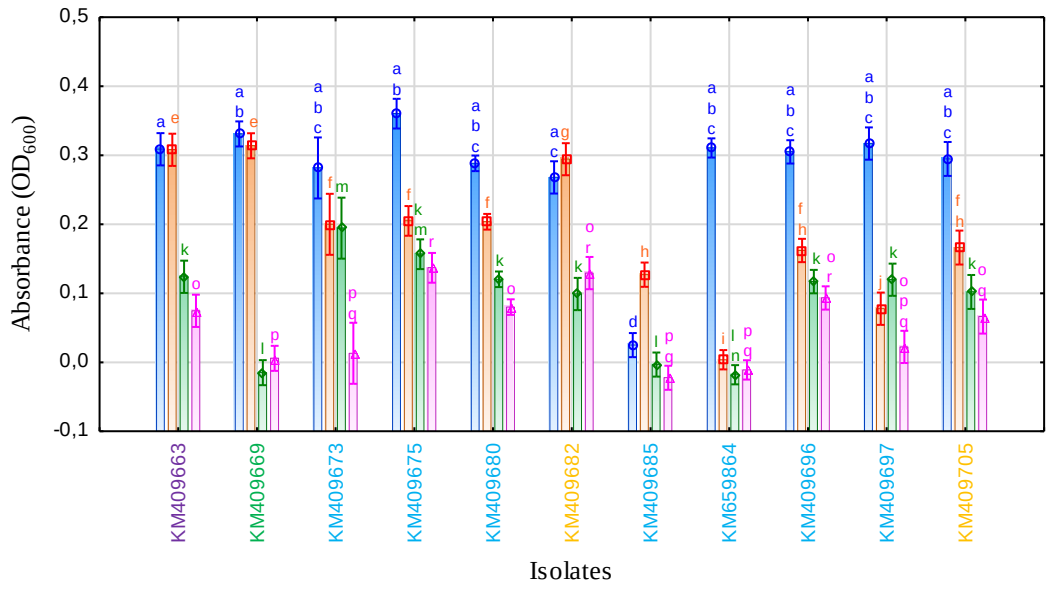

Figure 2.8: Graph showing mean (±SEM) growth of bacterial isolates from South African trout (**Farm B, Farm D, Farm E, Farm F**), grown at different salt concentration (**0%; 3.5%; 5%; 6.5%**). Different alphabetical letters denote a significant difference in growth of different isolates at a specific salt concentration.

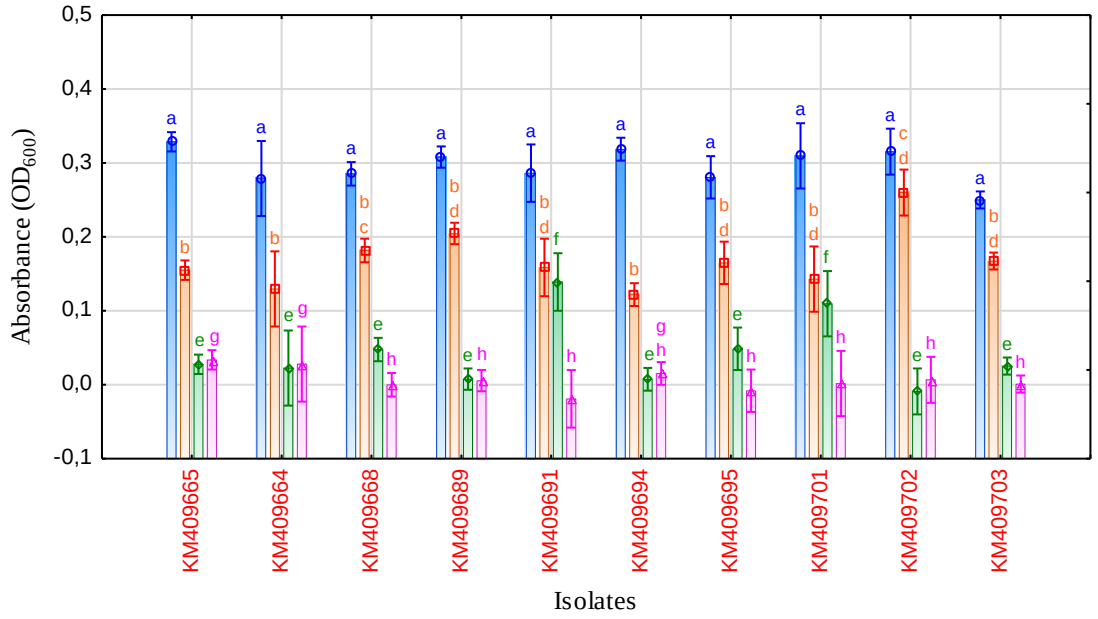

Figure 2. 9: Graph showing bacterial mean (±SEM) growth of isolates from Lesotho (**Farm A**) grown at different salt concentration (**0%; 3.5%; 5%; 6.5%**). Different alphabetical letters denote a significant difference in growth of different isolates at a specific salt concentration.

The results obtained for carbohydrate metabolism using the API 50CH strips, obtained from BioMérieux, Inc., are summarized in Table 2.3. Among the fifteen *L. garvieae* isolates tested, it was not possible to identify the strains to species level as *L. garvieae*, utilizing the

kit. However, biochemical differences were observed in this study amongst the *L. garvieae* isolates and between the *L. garvieae* isolates and *Lactococcus* isolate KM409665. More specifically, varied differences were noted among all our *L. garvieae* isolates in the assimilation of glycerol, L-arabinose, D-maltose, D-lactose, D-tagatose, potassium gluconate, amidon (starch) (isolate KM409664), D-saccharose sucrose and D-melobiose. Conversely, similarities were shared among all our *L. garvieae* isolates in response to assimilation of potassium 5-ketogluconate, potassium 2-ketogluconate, L-arabitol, D-arabitol, L-fucose, D-fucose, D-lyxose, D-turanose, gentiobiose, xylitol, glycogen, D-raffinose, D-melezitose, inulin and D-trehalose. In a study conducted by Bragg & Broere, (1986), where a *Streptococcus* species had been isolated from a diseased rainbow trout and was also characterized using API 50 CHS strips, the isolate fermented ribose, galactose, D-glucose, D-fructose, D-mannose, n-acetylglucosamine, amygdaline, arbutine, esculine, salicine, cellobiose, trehalose, β-gentiobiose and D-tagatose. However, none of the biochemical profiles documented in the previous study matched the profiles documented for any of the known species of *Streptococcus* on the API database (Bragg and Broere, 1986). Similarly, in our study, none of the *L. garvieae* isolates were identified by the kit as *L. garvieae*. The API 50CH kit was however able to identify the *Lactococcus* isolate KM409665 as *L. lactis subsp. lactis*. One notable result however, was between two isolates: *L. garvieae* KM409664 and *Lactococcus* isolate KM409665 with regards to D-lactose (bovine origin) and amidon (starch) assimilation. Both isolates were isolated from Lesotho at the same date. This was notable because the rest of the tested *L. garvieae* isolates were negative in both tests.

Table 2.3: API 50CH results for 14 *L. garvieae* isolates including the *Lactococcus* isolate KM409665.

Test	Test number	KM409685	KM409682	KM409668	KM409696	KM409680	KM409669	KM409689	KM409701	KM409673	KM409663	KM409695	KM409703	KM659864	KM409664	*Lactococcus* sp KM409665
Glycerol	1	-	-	-	-	-	?	-	-	?	?	-	?	-	?	-
Erythritol	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D- Arabinose	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L- Arabinose	4	-	-	-	-	-	-	-	-	-	-	?	-	?	-	-
D- Ribose	5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D- Xylose	6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
L- Xylose	7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D- Adonitol	8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Methyl- βD-Xylopyranoside	9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

D- Galactose	10	?	?	?	?	?	?	?	?	?	?	?	?	?	?	+
D- Glucose	11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D- Fructose	12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D- Mannose	13	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L- Sorbose	14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L- Rhamnose	15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Dulcitol	16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Inositol	17	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D- Mannitol	18	-	?	?	?	?	?	?	?	?	?	?	?	?	?	+
D- Sorbitol	19	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Methyl- αD-Mannopyranoside	20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Methyl- αD-Glucopyranoside	21	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
N- AcetylGlucosamine	22	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Amygdalin	23	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Arbutin	24	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Esculin ferric citrate	25	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Salicin	26	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D- Cellobiose	27	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D- Maltose	28	-	?	?	+	?	?	?	?	?	?	?	?	+	?	+
D- Lactose (bovine origin)	29	-	-	-	-	-	-	-	-	-	-	-	-	-	?	?
D- Melibiose	30	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D- Saccharose (sucrose)	31	-	+	+	-	-	+	+	+	+	+	+	+	-	+	+
D- Trehalose	32	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Inulin	33	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D- MeleZitose	34	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D- Raffinose	35	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Amidon (starch)	36	-	-	-	-	-	-	-	-	-	-	-	-	-	?	?
Glycogen	37	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Xylitol	38	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gentiobiose	39	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-TURanose	40	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-LYXose	41	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-TAGatose	42	+	+	+	-	-	+	+	+	+	+	+	+	-	-	-
D-FUCose	43	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-FUCose	44	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Arabitol	45	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Arabitol	46	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Potassium GlucoNaTe	47	-	?	?	?	?	?	?	?	?	?	?	?	?	?	?
Potassium 2-KetoGluconate	48	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Potassium 5-KetoGluconate	49	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

NB: Negative (-); Positive (+); Doubtful (?), **Farm B**, **Farm D**, **Farm E**, **Farm F**.

Oxytetracycline is the only antibiotic approved for use in South Africa aquaculture. Only eight *L. garvieae* isolates were susceptible to oxytetracycline, while 55% (n=11) had intermediate sensitivity. Our findings were similar to the results reported by Huchzemeyer & Henton (2011), who concluded that oxytetracycline is not as effective in treating fish as other antibiotics, such as amoxicillin and erythromycin, which is of concern since both antibiotics are not approved for use in South Africa. This would therefore make oxytetracycline a poor option for treatment of lactococcosis on trout farms in South African and Lesotho. In a study by Bragg & Broere (1986), the tested *Streptococcus* isolates were found to be sensitive to tetracycline, chloramphenicol and erythromycin, whereas the same isolates were found to be resistant to penicillin, clindamycin, amikacin, cotrimoxazole and gentamycin. In our study, penicillin G, chloramphenicol and vancomycin were shown to be most effective in preventing growth of *L. garvieae* isolates, whereas more than 55% of the isolates were resistant to sulfamethoxazole (n=11), norfloxacin (n=12), cefotaxime (n=12), kanamycin (n=11) and gentamycin (n=11). In a study by Sharifiyazdi et al. (2010), the *L. garvieae* strains tested in their study were also shown to be highly susceptible to chloramphenicol and erythromycin. The *Lactococcus* isolate KM409665 tested in the present study was shown to be susceptible to penicillin G, streptomycin, gentamycin, erythromycin and chloramphenicol. An intermediate reactivity of this isolate was recorded against kanamycin and vancomycin, whereas this isolate was shown to be resistant to cefotaxime, sulfamethoxazole, norfloxacin and oxytetracycline.

Table 2.4: Antimicrobial susceptibility test results for 20 *L. garvieae* isolates and *Lactococcus* isolate KM409665.

	***L. garvieae* (n= 20)**			***Lactococcus* isolate KM409665**		
Antibiotics	**R**	**I**	**S**	**R**	**I**	**S**
Penicillin G (10 mg/ml)	0	1	19	0	0	1
Streptomycin (50 mg/ml)	6	7	7	0	0	1
Gentamicin (10 mg/ml)	11	4	5	0	0	1
Erythromycin (15 mg/ml)	3	8	9	0	0	1
Chloramphenicol (34 mg/ml)	2	3	15	0	0	1
Kanamycin (50 mg/ml)	11	1	8	0	1	0
Cefotaxime (10 mg/ml)	12	3	5	1	0	0
Sulfamethoxazole (25 mg/ml)	11	8	1	1	0	0
Norfloxacin (10 mg/ml)	12	6	2	1	0	0
Oxytetracycline (30 mg/ml)	1	11	8	1	0	0
Vancomycin HCL (100 mg/ml)	0	0	20	0	1	0

R= resistant, I= intermediate, S= susceptible.

2.3.3 Carnobacterium maltaromaticum

Infections caused by *C. maltaromaticum* in spawning rainbow trout, including coho salmon (*O. kisutch*), steelhead trout (*O. mykiss*) and Chinook salmon (*O. tshawytscha*), had been reported in Michigan by Loch et al. (2011). Findings in their study demonstrated that infections with *C. maltaromaticum* are widespread in feral and captive *Oncorhynchus* sp. in Michigan and that phenotypic variations occurred amongst the isolates. The isolates in this study were identified as Gram-positive rods that grew very well on TSA. Bacterial colonies on TSA were pinpoint white, round, entire and on BA plates were circular, entire, pulvinate and whitish colonies ranging between 1.2-2 mm in diameter (Fig 2.11). The three isolates (KM409658, KM409660 and KM409659) identified as *C. maltaromaticum* clustered together with three *C. maltaromaticum* isolates in the phylogenetic analysis: *C. maltaromaticum* B26 (AM179873) (Fig.2), previously isolated from a rainbow trout intestine and considered to be part of the normal 'healthy' intestinal flora of trout (Kim & Austin, 2008); *C. maltaromaticum* MMF14 (GQ304922), isolated from a chinook salmon with no clinical signs

of disease (Loch et al., 2011) and *C. maltaromaticum* MF204 (AY543031) isolated from modified-atmosphere packaged (MAP) salmon (Rudi et al., 2004). This finding could be an indication that these three isolates may have been part of the normal flora of rainbow trout or may have been opportunistic pathogens that caused an infection during handling. Further investigations in to this matter will have to be performed.

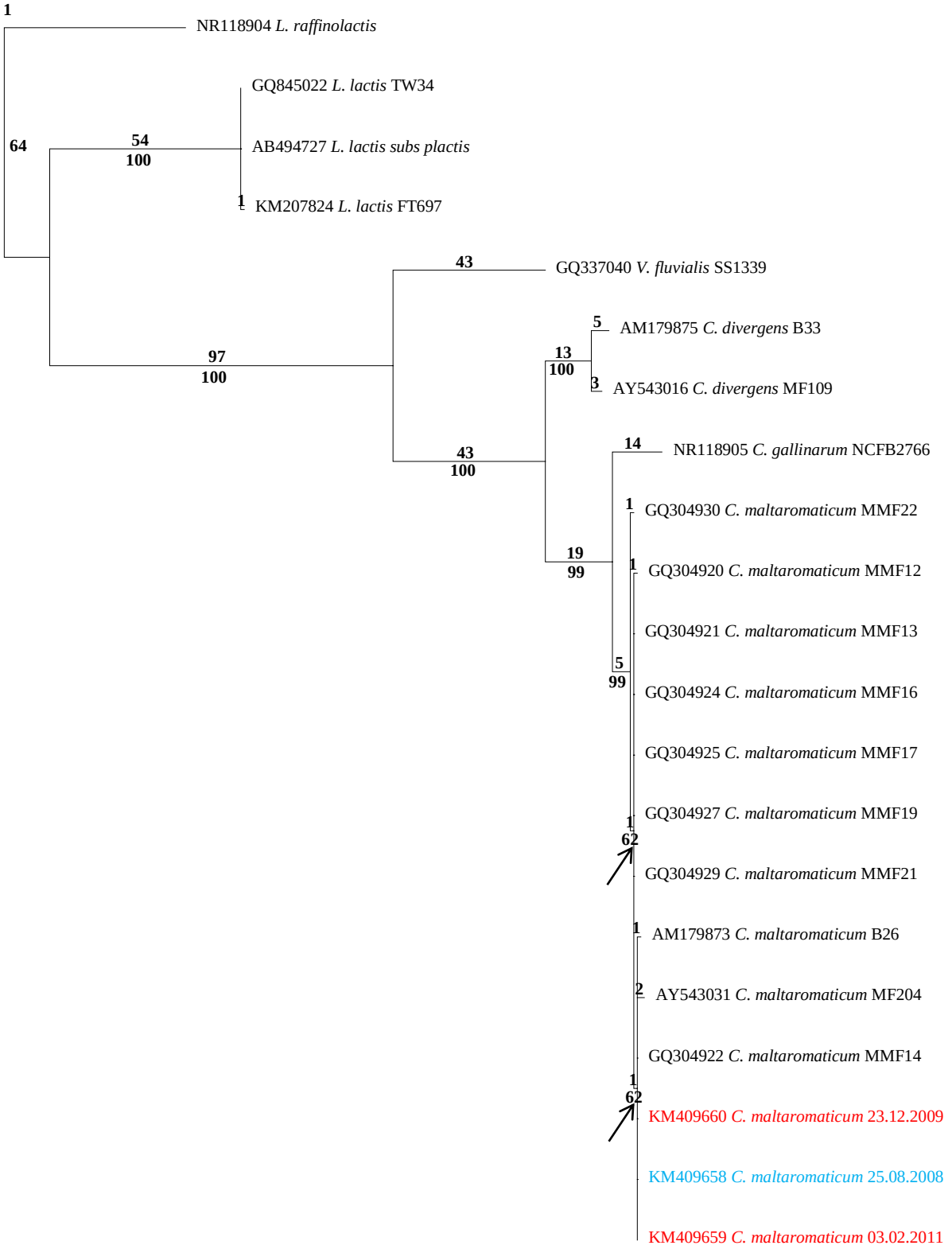

Figure 2.10: One of the shortest trees from the heuristic search using parsimony of *Carnobacterium* 16S rRNA gene sequences. Results revealed 15 trees retrieved with a total length of 368. The alignment matrix consisted of 1267 (80%) constant, 169 (11%) parsimony informative and 137 (9%) parsimony uninformative characters (CI= 0.916, RI= 0.950). Branch lengths are shown above branches, bootstrap percentages shown below branches and branches that collapse in the strict consensus indicated with an arrow. The colours relate to their origin (**Farm A**, **Farm B**, **Farm C**, **Farm D**, **Farm E**, **Farm F** and **Farm G**).

C. maltaromaticum strains require genotypic analysis to achieve a more definitive identification. *C. maltaromaticum* is a Gram-positive, facultative anaerobic, non-motile, non-spore-forming short to slender rod-shaped lactic acid bacterium. It is oxidase and catalase **negative.**

C. maltaromaticum is said to grow optimally at 30 ºC, whereas no growth occurs at temperatures above 40 ºC and growth decreases at temperatures below 15°C. The optimal pH at which *C. maltaromaticum* grows is between 6.0-7.0 (Buller, 2014). The three *C. maltaromaticum* isolates from this study showed similar characteristics to other *C. maltaromaticum* isoaltes, exhibiting delayed growth at 10 ºC, with an OD above 0.3, significantly similar growth after 24 hrs (Figure 2.12) and isolates continued to grow even after 48 hrs reaching an OD_{600} between 0.6-0.9. The latter isolates grew best at pH above 7, with no growth occurring at pH 4.5 and delayed growth recorded at pH of 5 (Figure 2.13). All three *C. maltaromaticum* species were sensitive to increasing salt concentration (see Figure 2.14) and had γ-haemolytic activity (Fig. 2.11).

(a) **(b)**

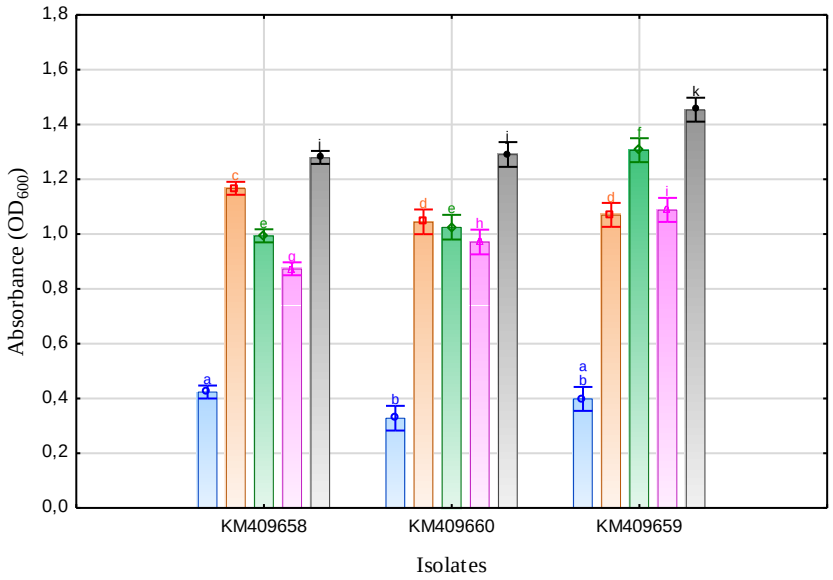

Figure 2.11: Graph showing the mean (±SEM) of *C. maltaromaticum* growth at different temperatures (**10 °C, 24 °C, 30 °C, 37 °C, 45 °C**). The different alphabetical letters denote a significant difference in growth of different isolates at a specific temperature.

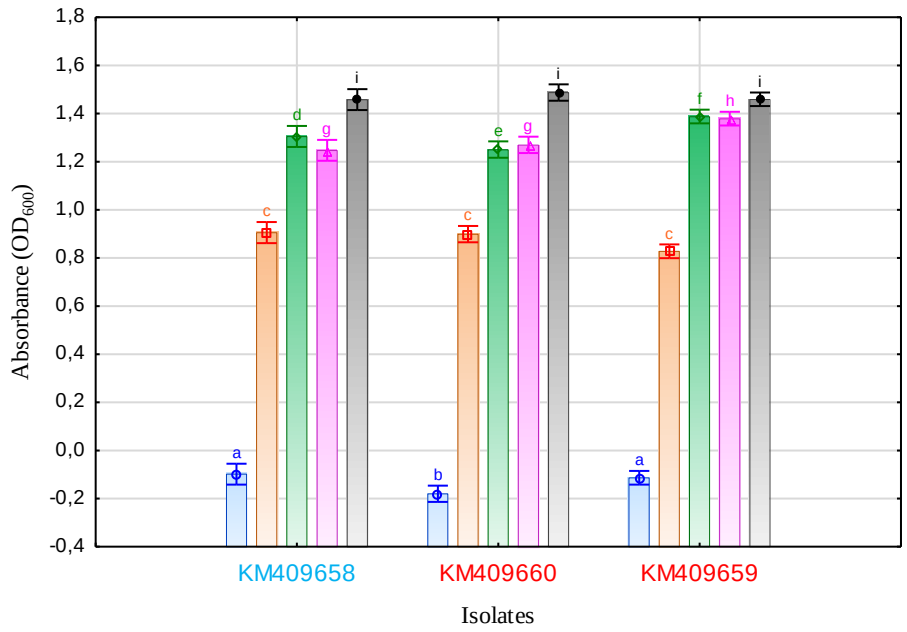

Figure 2.12: Graph showing the mean (±SEM) growth of *C. maltaromaticum* grown at different pH (**pH 4.5**, **pH 5**, **pH 7**, **pH 8**, **pH 9.5**). The different alphabetical letters denote a significant difference in growth of different isolates at a specific pH.

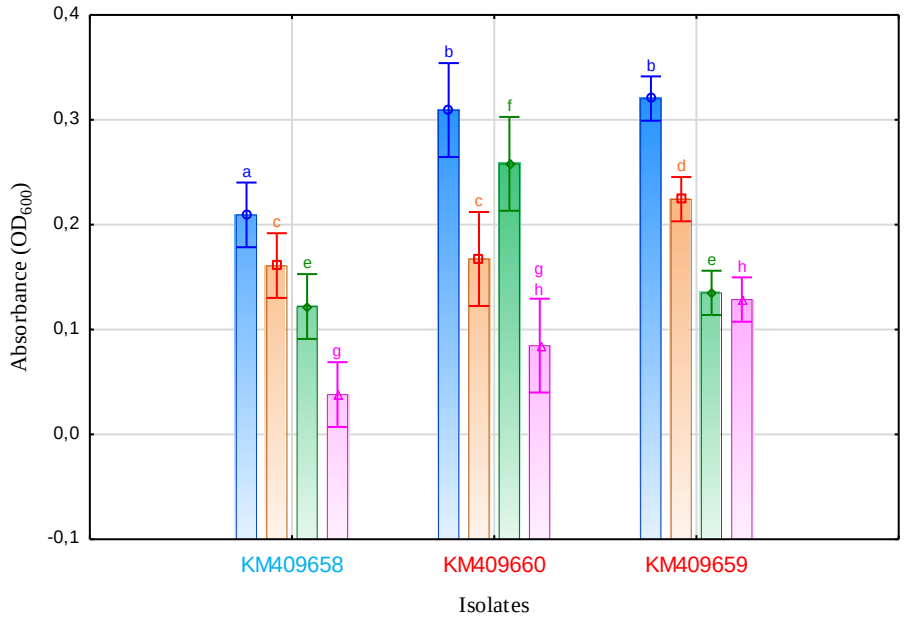

Figure 2.13: Graph showing the mean (±SEM) growth of *C. maltaromaticum* isolates (0%, 3.5%, 5%, 6.5%) at different salt concentrations. The different alphabetical letters denote a significant difference in growth of different isolates at a specific NaCl.

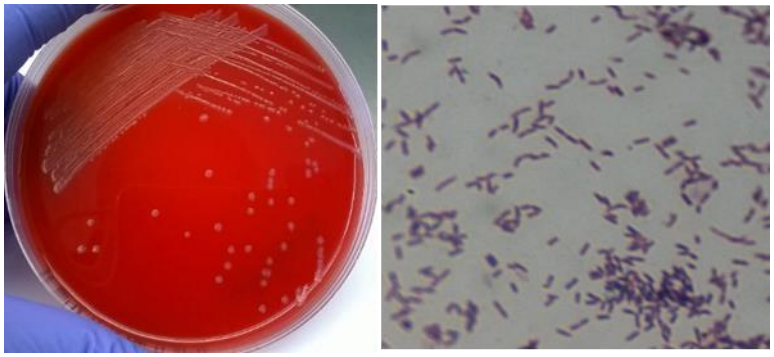

Figure 2.14: *C. maltaromaticum* on blood agar plate (a) representing γ-haemolysis, (b) showing Gram positive rods.

Intraspecies differences were noted among the *C. maltaromaticum* strains with regards to assimilation of D-galactose, D-mannitol, methyl-αD-glucopyranoside, D-melibiose, inulin and D-melezitose.

Table 2.5: API 50CH results for all *C. maltaromaticum* isolates tested.

Test	Test number	KM409658	KM409660	KM409659	Test	Test number	KM409658	KM409660	KM409659	Test	Test number	KM409658	KM409660	KM409659
Glycerol	1	?	?	?	D- Mannitol	18	-	?	+	D- Raffinose	35	-	-	-
Erythritol	2	-	-	-	D- Sorbitol	19	-	-	-	Amidon (starch)	36	?	?	?
D- Arabinose	3	-	-	-	Methyl- αD-Mannopyranoside	20	-	-	-	Glycogen	37	-	-	-
L- Arabinose	4	-	-	-	Methyl- αD-Glucopyranoside	21	?	+	?	Xylitol	38	-	-	-
D- Ribose	5	+	+	+	N-AcetylGlucosamine	22	+	+	+	Gentiobiose	39	+	+	+
D- Xylose	6	-	-	-	Amygdalin	23	+	+	+	D-TURanose	40	?	?	?
L- Xylose	7	-	-	-	Arbutin	24	+	+	+	D-LYXose	41	-	-	-
D- Adonitol	8	-	-	-	Esculin ferric citrate	25	+	+	+	D-TAGatose	42	-	-	-
Methyl- βD-Xylopyranoside	9	-	-	-	Salicin	26	+	+	+	D-FUCose	43	-	-	-
D- Galactose	10	?	-	?	D- Cellobiose	27	+	+	+	L-FUCose	44	-	-	-
D- Glucose	11	+	+	+	D- Maltose	28	+	+	+	D-Arabitol	45	-	-	-
D- Fructose	12	+	+	+	D- Lactose (bovine origin)	29	-	-	-	L-Arabitol	46	-	-	-
D- Mannose	13	+	+	+	D- Melibiose	30	?	-	-	Potassium GlucoNaTe	47	?	?	?
L- Sorbose	14	-	-	-	D- Saccharose (sucrose)	31	+	+	+	Potassium 2-KetoGluconate	48	-	-	-
L- Rhamnose	15	-	-	-	D- Trehalose	32	+	+	+	Potassium 5-KetoGluconate	49	-	-	-
Dulcitol	16	-	-	-	Inulin	33	-	?	-					
Inositol	17	-	-	-	D- MeleZitose	34	?	-	?					

NB: Negative (-); Positive (+); Doubtful (?)

All three *C. maltaromaticum* strains were susceptible to penicillin G, erythromycin, chloramphenicol, oxytetracycline and vancomycin, but resistant to cefotaxime and

sulfamethoxazole. They varied in reactivity between streptomycin, gentamycin, kanamycin and norfloxacin (table 2.6).

Table 2. 6: Antimicrobial susceptibility results of the three *C. maltaromaticum* isolates.

***C. maltaromaticum* (n= 3)**			
Antibiotics	**R**	**I**	**S**
penicillin G (10 mg/ml)	0	0	3
streptomycin (50 mg/ml)	2	1	0
gentamycin (10 mg/ml)	0	2	1
erythromycin (15 mg/ml)	0	0	3
chloramphenicol (34 mg/ml)	0	0	3
kanamycin (50 mg/ml)	0	1	2
cefotaxime (10 mg/ml)	3	0	0
sulfamethoxazole (25 mg/ml)	3	0	0
norfloxacin (10 mg/ml)	0	1	2
oxytetracycline (30 mg/ml)	0	0	3
vancomycin HCL (100 mg/ml)	0	0	3

R= resistant, I= intermediate, S= susceptible.

2.3.4 Weissella species.

Weissella species were first proposed by Collins et al. (1993) based on 16S rRNA gene phylogenetic analysis. The first disease outbreak caused by *Weissella* species on rainbow trout was reported in China in 2009, where six Gram-positive rods identified as *Weissella* species were isolated from diseased adult rainbow trout during the winter months of 2007 (Liu et al., 2009). In 2008 and 2009, some commercial rainbow trout farms in Brazil experienced an outbreak of weissellosis. The phylogenetic analysis carried out in the latter study indicated 100% bootstrap support with the 2007 strains isolated from China (Figueiredo et al., 2012). A third case was reported in the US, Western North Carolina, at a commercial rainbow trout farm during the summer of 2011 (Welch & Good., 2013). Again, the sequences of these isolates were 99% similar with the Chinese and Brazilian isolates. Based on the phylogeny analysis done in this study, the two isolates from South African trout (KM409656

and KM409657), were identified as *Weissella* species and formed a distinct cluster with a 91% bootstrap support (Fig 2.15) with all the *Weissella* species previously isolated from diseased rainbow trout farmed in Brazil, United States and China. These *Weissella* species appear to be closely related to a *W. ceti* 11191A09 (NR117039) strain that was isolated from the spleen of a beaked whale in Spain (Vela et al., 2011), forming a single cluster with a 100% bootstrap support. In a study by Liu et al., (2009), the same *W. ceti* 11191A09 strain showed a 99.2–99.5% sequence similarity to the unidentified *Weissella* species from diseased trout cultured in China. This finding clearly indicates that all these recently identified *Weissella* species are very closely related to *W. ceti*.

Weissella species are non-motile, non-spore forming Gram-positive cocci, are catalase and oxidase negative, with variable α-haemolysis on BA. Growth occurs on MRS, but not on MacConkey agar. Growth occurs at 20–45 °C (Buller, 2014; Figueiredo et al., 2012). *W. ceti* on the other hand is a Gram-positive coccoid to short rod (1.5×0.2 µm) shaped bacterium, and is non-pigmented with an α-haemolysis on BA plates. Growth occurs at 22–37 °C, but not at or below 15 and above 42 °C. Growth also occurs at NaCl concentrations ranging from 3–6.5% (Buller, 2014).

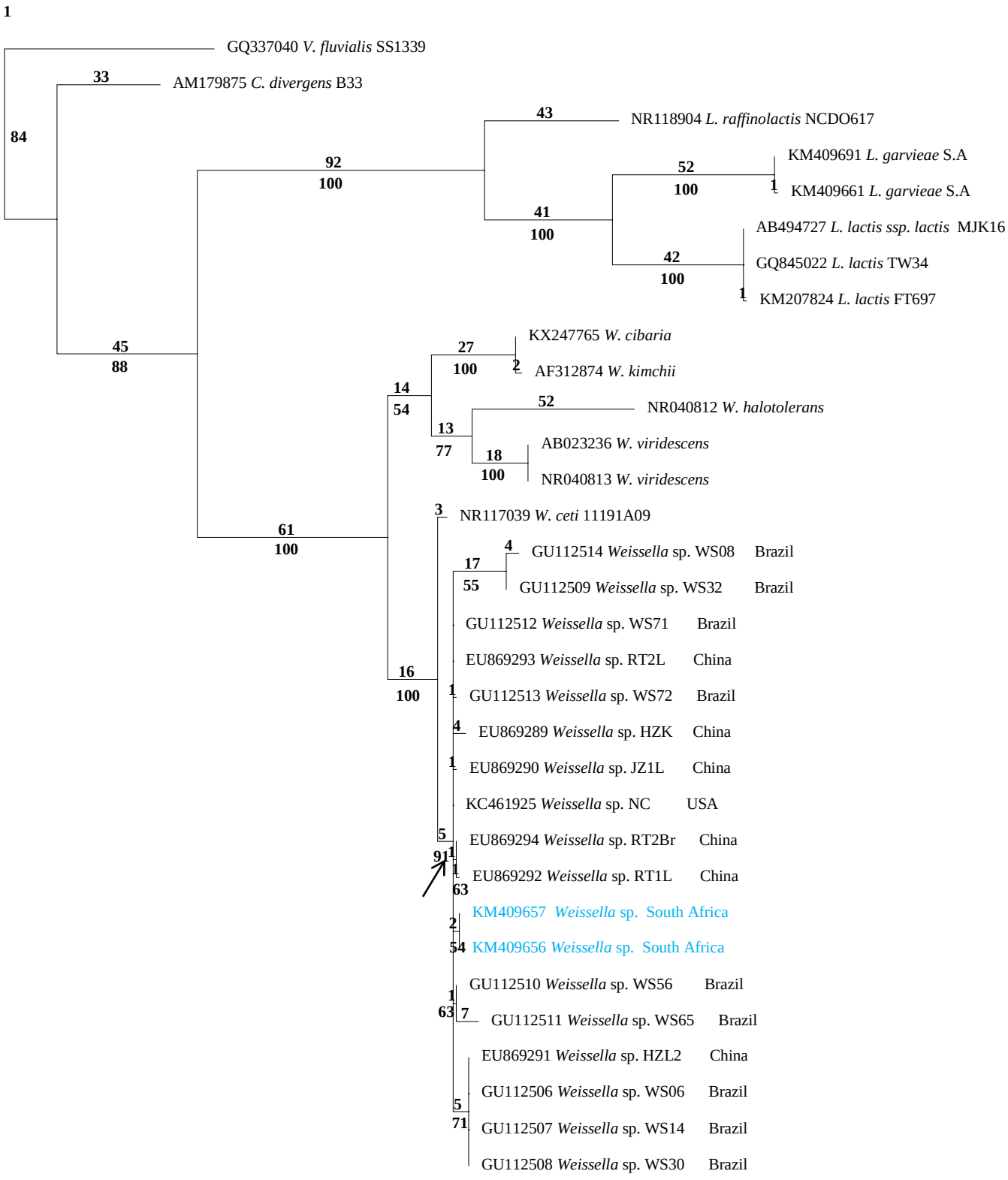

Figure 2.11: One of the shortest trees retrieved from the heuristic search using parsimony of *Weissella* species 16S rRNA gene sequences from South Africa (KM409657 & KM409656), China and Brazil. Results revealed 10 trees with a total length of 1011. The alignment matrix consisted of 1088 (68%) constant, 286 (18%) parsimony informative and 218 (14%) parsimony uninformative (CI= 0.750, RI= 0.878) characters. Branch lengths are shown above branches, bootstrap percentages shown below branches and branches that collapse in the strict consensus indicated with an arrow. The colours relate to their origin (**Farm A, Farm B, Farm C, Farm D, Farm E, Farm F and Farm G**).

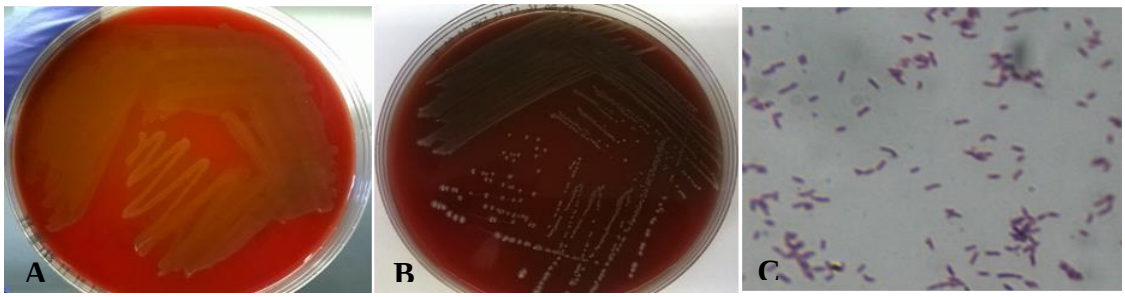

Figure 2.12: *Weissella* isolates (A & B = α- haemolysis on BA), C= Gram-positive rods.

The phenotypic and physiological analysis performed in our study confirmed the two Weissella isolates to be different from the rest of the isolates assessed in this study. Both isolates were characterized to be Gram-positive, short rods that were catalase and oxidase negative. On TSA plates, the two *Weissella* isolates grew poorly, forming very small circular, convex and translucent (non-pigmented) colonies of approximately 1 mm diameter. Growth was observed best on BA plates with colonies ranging between 0.8-1.5 mm diameter, showing an α-heamolysis on BA plates, (Fig 2.15). This is, to the best of our knowledge, the first report of weisselliosis in rainbow trout from South Africa.

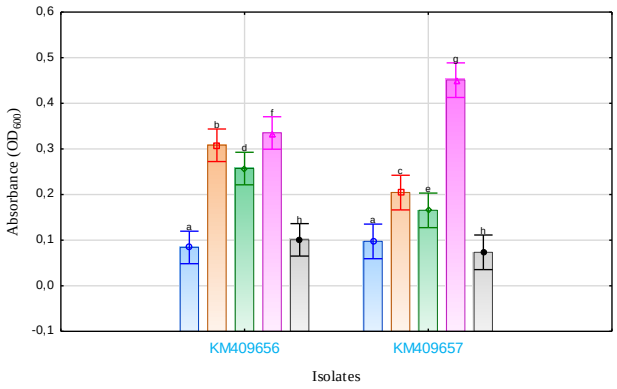

Figure 2.13: Graph representing the mean (±SEM) growth of the two *Weissella* isolates. at different temperatures (**10 °C, 24 °C, 30 °C, 37 °C, 45 °C**). The different alphabetical letters denote a significant difference in growth of different isolates at specific temperatures.

Current literature suggests that growth of *Weissella* species is optimal between 20-45 ºC (Buller, 2014), which confirms findings from this study. Both isolates in this study did not grow at 10 ºC and 45 ºC, but cells remained viable even after 48 hrs of incubation with an OD600 ≤ 0.1. Furthermore, there was no significant difference between the growth of the two isolates at 10 °C (p= 0,680246) and 45 °C (p= 0,202898). The best growth occurred at 37 ºC, followed by 24 °C (Fig 2.17).

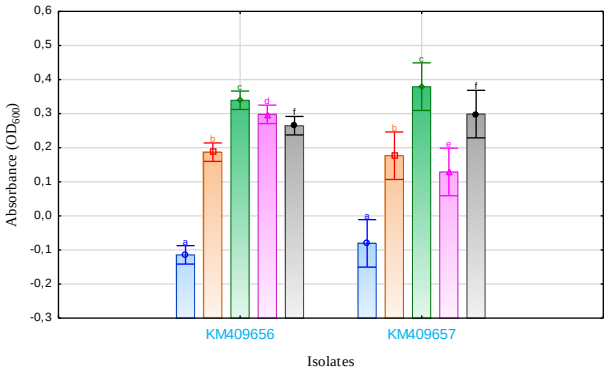

Figure 2.14: Graph representing the growth of the two *Weissella* isolates at different pH (**pH 4.5, pH 5, pH 7, pH 8, pH 9.5**). The different alphabetical letters denote a significant difference in growth of different isolates at different pH values.

Both *Weissella* isolates could not grow at pH 4.5 and delayed growth occurred at pH 5. Best growth occurred at pH ranging from 7 to 9.5.

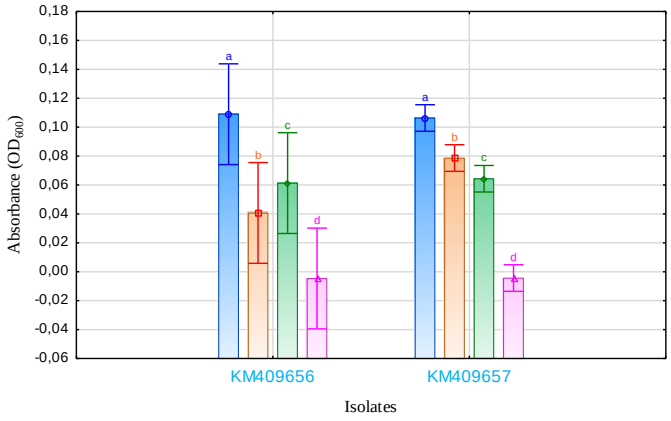

Figure 2.15: Graph representing the growth of the two *Weissella* isolates at different salt concentrations (**0%, 3.5%, 5%, 6.5%**). The different alphabetical letters denote a significant difference in growth of different isolates at different NaCl concentrations.

Both *Weissella* isolates struggled to grow in the presence of NaCl. Growth was only recorded when no NaCl was present, with no cell growth occurring in media supplemented with 3.5-6.5% NaCl. For both the pH and salinity analysis, no significant difference in growth ($p > 0.05$) was recorded between the two *Weissella* isolates tested in this study.

Table 2.7: API 50CH results of the two *Weissella* isolates.

Test	Test number	KM409657	KM409656	Test	Test number	KM409657	KM409656	Test	Test number	50 KM409657	53 KM409656
Glycerol	1	-	-	D- Mannitol	18	-	-	D- Raffinose	35	-	-
Erythritol	2	-	-	D- Sorbitol	19	-	-	Amidon (starch)	36	-	-
D- Arabinose	3	-	-	Methyl- αD-Mannopyranoside	20	-	-	Glycogen	37	-	-
L- Arabinose	4	-	-	Methyl- αD-Glucopyranoside	21	-	-	Xylitol	38	-	-
D- Ribose	5	+	+	N-AcetylGlucosamine	22	-	-	Gentiobiose	39	-	-
D- Xylose	6	-	-	Amygdalin	23	-	-	D-TURanose	40	-	-
L- Xylose	7	-	-	Arbutin	24	-	-	D-LYXose	41	-	-
D- Adonitol	8	-	-	Esculin ferric citrate	25	+	+	D-TAGatose	42	-	-
Methyl- βD-Xylopyranoside	9	-	-	Salicin	26	-	-	D-FUCose	43	-	-
D- Galactose	10	-	-	D- Cellobiose	27	-	-	L-FUCose	44	-	-
D- Glucose	11	-	-	D- Maltose	28	-	-	D-Arabitol	45	-	-
D- Fructose	12	-	-	D- Lactose (bovine origin)	29	-	-	L-Arabitol	46	-	-
D- Mannose	13	-	-	D- Melibiose	30	-	-	Potassium GlucoNaTe	47	-	-
L- Sorbose	14	-	-	D- Saccharose (sucrose)	31	-	-	Potassium 2-KetoGluconate	48	-	-
L- Rhamnose	15	-	-	D- Trehalose	32	-	-	Potassium 5-KetoGluconate	49	-	-
Dulcitol	16	-	-	Inulin	33	-	-				
Inositol	17	-	-	D- MeleZitose	34	-	-				

NB: Negative (-); Positive (+); Doubtful (?)

The two isolates from this study could not be identified as *Weissella* isolates using the API 50CH kit. Both isolates were shown to be positive for D-ribose and esculin hydrolysis. In a study done by Welch & Good (2013), their *Weissella* strains had negative reactions for esculin hydrolysis, whereas our isolates were positive on the API 50CH kit. Their isolates were also found to produce acid from glycogen, D-ribose and D-trehalose and did not produce acid from L-arabinose, D-mannitol, D-sorbitol, D-lactose, D-raffinose, inulin, β-galactosidase, β-glucuronidase, α-galactosidase and pyrrolidonyl arylamidase, while our isolates were only positive for D-ribose. In overall, both isolates were only positive for D-Ribose and esculin ferric citrate.

Table 2.8: Antimicrobial susceptibility results of the two *Weissella* species.

***Weissella species* (n= 2)**			
Antibiotics	**R**	**I**	**S**
penicillin G (10 mg/ml)	0	0	2
streptomycin (50 mg/ml)	0	1	1
gentamycin (10 mg/ml)	1	1	0
erythromycin (15 mg/ml)	0	2	0
chloramphenicol (34 mg/ml)	1	0	1
kanamycin (50 mg/ml)	2	0	0
cefotaxime (10 mg/ml)	2	0	0
sulfamethoxazole (25 mg/ml)	2	0	0
norfloxacin (10 mg/ml)	0	2	0
Oxytetracycline (30 mg/ml)	0	0	2
Vancomycin HCL (100 mg/ml)	1	1	0

R= resistance, I= intermediate, S= susceptible.

The antimicrobial susceptibility results for the two *Weissella* isolates tested in this study are listed in table 2.8. Both *Weissella* isolates were susceptible to penicillin G and oxytetracycline. An intermediate sensitivity to erythromycin and norfloxacin was observed and complete resistance to kanamycin, cefotaxime and sulfamethoxazole by both isolates. The two *Weissella* isolates had varied sensitivity to several antibiotics, including streptomycin, gentamycin, chloramphenicol and vancomycin. Therefore, oxytetracycline could still be used for treating infections caused by these *Weissella* isolates, given the list of drugs approved for use within South African trout aquaculture (table 1.2).

Table 2.9: Overall results obtained for phenotype, biochemical, and physiological analysis performed in this study.

Strain with Genbank Accession Number, Isolation date	Colony morphology	Gram staining	Motility	Oxidase	Catalase	Growth on		Temperature ()					Salinity ()				pH				
						TSA	4 BA	10	24	30	37	45	0	3.5	5	6.5	4.5	5	7	8	9.5
Isolates obtained from trout farmed in South Africa																					
2006																					
L. garvieae KM409685	11.07.2006	Entire, undulate,whitish ≈2mm	cocci (+)	—	—	—	+	γ	+ dg	+	+	+	+ dg	—	+ dg	—	—	—	+	+	+	+
2007																					
L. garvieae KM409683	13.12.2007	Entire, undulate,whitish ≈2mm	cocci (+)	n/a	—	—	+	α	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
2008																					
L. garvieae KM409682	07.01.2008	Entire, undulate,whitish ≈2mm	cocci (+)	—	—	—	+	α	+ dg	+	+	+	+	+ dg	+ dg	RV	+ dg	—	+	+	+	+
L. garvieae KM409705	25.08.2008	Entire, undulate,whitish ≈2mm	cocci (+)	—	—	—	+	γ	RV	+	+	+	+	+ dg	+ dg	RV	—	+	+	+	+	+
C. maltaromaticum KM409658	25.08.2008	Pulvinate,whitish 1.2-2mm	rod (+)	—	—	—	+	γ	+ dg	+	+	+	+	+ dg	+ dg	+ dg	—	—	+ dg	+	+	+
L. garvieae KM409696	22.09.2008	Entire, undulate,whitish ≈2mm	cocci (+)	—	—	—	+	α	+ dg	+	+	+	+	+ dg	+ dg	+ dg	RV	+	+	+	+	+
L. garvieae KM409675	19.11.2008	Entire, undulate,whitish ≈2mm	cocci (+)	n/a	—	—	+	γ	+ dg	+	+	+	+	+	+	+	+ dg	+	+	+	+	+
L. garvieae KM409676	19.11.2008	Entire, undulate,whitish ≈2mm	cocci (+)	n/a	—	—	+	γ	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
L. garvieae KM409677	01.12.2008	Entire, undulate,whitish ≈2mm	cocci (+)	n/a	—	—	+	γ	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
L. garvieae KM409678	01.12.2008	Entire, undulate,whitish ≈2mm	cocci (+)	n/a	—	—	+	γ	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
L. garvieae KM409679	01.12.2008	Entire, undulate,whitish ≈2mm	cocci (+)	n/a	—	—	+	γ	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
L. garvieae KM409667	26.06.2008	Entire, undulate,whitish ≈2mm	cocci (+)	n/a	—	—	+	γ	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
L. garvieae KM409684	26.06.2008	Entire, undulate,whitish ≈2mm	cocci (+)	n/a	—	—	+	γ	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
L. garvieae KM409706	05.09.2008	Entire, undulate,whitish ≈2mm	cocci (+)	n/a	—	—	+	γ	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
L. garvieae KM409707	05.09.2008	Entire, undulate,whitish ≈2mm	cocci (+)	n/a	—	—	+	γ	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
2009																					
L. garvieae KM409680	02.03.2009	Entire, undulate,whitish ≈2mm	cocci (+)	—	—	—	+	γ	+ dg	+	+	+	+	+ dg	+ dg	+ dg	—	—	+ dg	+	+	+
L. garvieae KM409673	22.10.2009	Entire, undulate,whitish ≈2mm	cocci (+)	—	—	—	+	γ	+ dg	+	+	+	+	+ dg	+ dg	+ dg	—	—	+ dg	+	+	+
L. garvieae KM409674	22.10.2009	Entire, undulate,whitish ≈2mm	cocci (+)	n/a	—	—	+	γ	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a

Positive (+), Negative (—), dg= delayed growth, n/a= not tested, RV= remained viable at 0.1nm. (**Farm A**, **Farm B**, **Farm C**, **Farm D**, **Farm E**, **Farm F** and **Farm G**)

Table 2.9: Continued

L. garvieae KM409670	19.03.2009	Entire, undulate,whitish ≈2mm	cocci (+)	n/a	—	—	+	γ	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
L. garvieae KM409671	19.03.2009	Entire, undulate,whitish ≈2mm	cocci (+)	n/a	—	—	+	γ	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
L. garvieae KM409669	25.03.2009	Entire, undulate,whitish ≈2mm	cocci (+)	–	—	—	+	α	+ dg	+	+	+	+	+	+	–	–	+ dg	+ dg	+	+	+
L. garvieae KM409672	21.10.2009	Entire, undulate,whitish ≈2mm	cocci (+)	n/a	—	—	+	γ	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
2010																						
L. garvieae KM409666	22.01.2010	Entire, undulate,whitish ≈2mm	cocci (+)	n/a	—	—	+	α	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
L. garvieae KM409662	11.03.2010	Entire, undulate,whitish ≈2mm	cocci (+)	n/a	—	—	+	γ	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
L. garvieae KM409663	11.03.2010	Entire, undulate,whitish ≈2mm	cocci (+)	–	—	—	+	γ	+ dg	+	+	+	+	+	+	+ dg	–	+ dg	+ dg	+	+	+
***L. garvieae* KM409681**	**30.03.2010**	Entire, undulate,whitish ≈2mm	cocci (+)	n/a	—	—	+	α	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
L. garvieae KM659863	17.08.2010	Entire, undulate,whitish ≈2mm	cocci (+)	n/a	—	—	+	α	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
2011																						
Weissella sp,KM409656	16.03.2011	Convex,translucent ≈1mm	rod (+)	—	—	—	+	α	+ dg	+	+	+	RV	+ dg	–	–	–	—	+ dg	+	+	+
Weissella sp,KM409657	11.04.2011	Convex,translucent ≈1mm	rod (+)	—	—	—	+	α	RV	+	+	+	RV	+ dg	RV	–	–	—	+ dg	+	+	+
L. garvieae KM409697	21.12.2011	Entire, undulate,whitish ≈2mm	cocci (+)	—	—	—	+	γ	+ dg	+	+	+	+	+	–	–	–	+	+ dg	+	+	+
L. garvieae KM409692	06.12.2011	Entire, undulate,whitish ≈2mm	cocci (+)	n/a	—	—	+	α	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
L. garvieae KM409693	06.12.2011	Entire, undulate,whitish ≈2mm	cocci (+)	n/a	—	—	+	α	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
2012																						
L. garvieae KM659864	08.02.2012	Entire, undulate,whitish ≈2mm	cocci (+)	–	–	–	+	α	+ dg	+	+	+	+	+	–	–	–	+	+	+	+	+
Isolates obtained from trout farmed in Lesotho																						
2008																						
L. garvieae KM409668	17.01.2008	Entire, undulate,whitish ≈2mm	cocci (+)	–	–	–	+	α	+ dg	+	+	+	+	+	+ dg	–	–	–	+ dg	+	+	+
2009																						
L. garvieae KM659862	15.01.2009	Entire, undulate,whitish ≈2mm	cocci (+)	n/a	–	–	+	α	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
L. garvieae KM409686	15.01.2009	Entire, undulate,whitish ≈2mm	cocci (+)	n/a	–	–	+	α	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Positive (+), Negative (–), dg= delayed growth, n/a= not tested, RV= remained viable at 0.1nm. (**Farm A**, **Farm B**, **Farm C**, **Farm D**, **Farm E**, **Farm F** and **Farm G**)																						n/a

Table 2.9: Continued

L. garvieae KM409687	15.01.2009	Entire, undulate,whitish ≈2mm	cocci (+)	n/a	—	—	+	γ	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	
L. garvieae KM409688	15.01.2009	Entire, undulate,whitish ≈2mm	cocci (+)	n/a	—	—	+	γ	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
L. garvieae KM409698	19.01.2009	Entire, undulate,whitish ≈2mm	cocci (+)	n/a	—	—	+	α	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
L. garvieae KM409699	19.01.2009	Entire, undulate,whitish ≈2mm	cocci (+)	n/a	—	—	+	γ	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
L. garvieae KM409700	19.01.2009	Entire, undulate,whitish ≈2mm	cocci (+)	n/a	—	—	+	α	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
L. garvieae KM409689	17.04.2009	Entire, undulate,whitish ≈2mm	cocci (+)	—	—	—	+	α	+ dg	+	+	+	+	+	+ dg	—	—	+	+	+	+	+
L. garvieae KM409701	17.04.2009	Entire, undulate,whitish ≈2mm	cocci (+)	—	—	—	+	α	—	+	+	+	+	+	+ dg	RV	—	—	+ dg	+	+	+
L. garvieae KM409702	17.04.2009	Entire, undulate,whitish ≈2mm	cocci (+)	—	—	—	+	γ	—	+	+	+	+	+	+ dg	—	—	—	+ dg	+	+	+
C. maltaromaticum KM409660	23.12.2009	Pulvinate,whitish 1.2-2mm	rod (+)	—	—	—	+	γ	+ dg	+	+	+	+	+	+ dg	+ dg	—	—	+	+	+	+
2010																						
L. garvieae KM409661	23.04.2010	Entire, undulate,whitish ≈2mm	cocci (+)	n/a	—	—	+	γ	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
2011																						
C. maltaromaticum KM409659	03.02.2011	Pulvinate,whitish 1.2-2mm	rod (+)	—	—	—	+	γ	+ dg	+	+	+	+	+	+ dg	+ dg	+ dg	—	+	+	+	+
L. garvieae KM409694	11.03.2011	Entire, undulate,whitish ≈2mm	cocci (+)	—	—	—	+	α	—	+	+	+	+	+	+ dg	—	—	—	+ dg	+	+	+
L. garvieae KM409695	11.03.2011	Entire, undulate,whitish ≈2mm	cocci (+)	—	—	—	+	α	—	+	+	+	+	+	+ dg	—	—	—	+ dg	+	+	+
L. garvieae KM409703	11.03.2011	Entire, undulate,whitish ≈2mm	cocci (+)	—	—	—	+	γ	—	+	+	+	+	+	+	—	—	—	+	+	+	+
L. garvieae KM409704	11.03.2011	Entire, undulate,whitish ≈2mm	cocci (+)	n/a	—	—	+	γ	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
2012																						
L. garvieae KM409690	18.01.2012	Entire, undulate,whitish ≈2mm	cocci (+)	n/a	—	—	+	α	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
L. garvieae KM409691	18.01.2012	Entire, undulate,whitish ≈2mm	cocci (+)	—	—	—	+	α	—	+	+	+	+	+	+ dg	+ dg	—	—	+	+	+	+
L. garvieae KM409664	13.02.2012	Entire, undulate,whitish ≈2mm	cocci (+)	—	—	—	+	α	—	+	+	+	+	+	+ dg	—	—	+	+	+	+	+
Lactococcus sp, KM409665	13.02.2012	Entire, undulate,whitish ≈2mm	cocci (+)	—	—	—	+	γ	+	+	+	+	+	+	+ dg	—	—	+	+	+	+	+

Positive (+), Negative (—), dg= delayed growth, n/a= not tested, RV= remained viable at 0.1nm. (**Farm A**, **Farm B**, **Farm C**, **Farm D**, **Farm E**, **Farm F** and **Farm G**)

CHAPTER 3: ANTIGENICITY ANALYSIS

3.1 INTRODUCTION

Pathogenic bacteria have developed strategies that enable them to multiply within their host and avoid recognition by the various components of the innate and the adaptive immune systems, allowing them to successfully colonize and establish an infection within their hosts (Deitsch et al., 2009). Several mechanisms are utilized to gain access to and cause disease within a host, including expression of a wide range of molecules that allows them to bind to the host cells to facilitate a variety of different host responses. This ability to invade and evade the host's immune system is brought about by virulence factors, such as invasion or evasion factors and endo- or exotoxins. These molecules are called antigenic determinants or Pathogen (or Microbe) - Associated Molecular Patterns (PAMPs/ MAMPs) (Janeway, 1989). PAMPs/MAMPs are unique to specific pathogens or conserved across several different species. Other PAMPs include microbial carbohydrates such as lipopolysaccharides, peptidoglycans, cell wall proteins, lipoteichoic acid, bacterial peptides such as flagellin, lipoproteins and nucleic acids (bacterial or viral DNA or RNA) (Mogensen, 2009).

The ability to avoid recognition and subsequent destruction by the host's immune cells also involves manipulation or alteration of structural characteristics of PAMPs, which is termed antigenic variation. There are two types of molecular mechanisms involved in antigenic variation: genetic mechanisms, such as mutation and recombination; and epigenetic mechanisms that affect the expression of a gene. Antigenic variation results in a heterogenic phenotype of a clonal population. Individual cells either express the antigen-variable protein(s) or express one of multiple antigenic forms of the protein. This form of regulation has been identified mainly, but not exclusively, for a wide variety of surface structures on pathogens and is implicated as a virulence strategy. The main benefit of antigenic variation is the ability to evade the developing or developed acquired immune response of the infected host, by interfering with the type of antibody produced during an initial infection phase. This specific immunity is developed against a subset of specific surface structures expressed by the infecting organism. If the infectious organism changes these surface structures to new variants, which could be as little as one or two amino acids differences of a particular protein, previous acquired immunity may be ineffective and reinfection can occur (Frank, 2002; Deitsch et al., 2009).

A key factor in the fight against bacterial disease, especially when selecting strains for vaccination, is the identification and characterization of all of the antigenic determinants of the causative pathogen. It appears that there is vast diversity amongst the 49 *L. garvieae* strains studied in this study, as indicated by results of the phylogenetic analysis of the 16S rRNA gene sequences conducted on the isolates in Chapter 2. However, the structural diversity of the cell surface antigens possessed by these strains remains unknown. The aim of this chapter was therefore not to specifically identify the antigenic determinants of each isolate, but rather to assess whether different *L. garvieae* serotypes exist within this population using six rabbit produced anti-*L. garvieae* polyclonal antibodies. This analysis would assist in selecting a minimum number of strains that may be suitable for the production of vaccine(s) that will hopefully provide effective protection against most, if not all, of the isolates examined in this study.

Rabbit produced polyclonal antibodies were selected for this study because they are inexpensive to produce and possess high affinity, due to the recognition of multiple epitopes. The first criteria used in the selection of strains for antibody production was based on the phylogenetic analysis (Fig 2.2) results obtained on Chapter 2. Firstly, one *Lactococcus* isolate KM409665 and one *L. garvieae* isolate KM409673, that were randomly chosen, were used for the vaccination of rabbits and antibodies were produced against the *Lactococcus* species. These antibodies were then used in cross-reactivity studies against all isolates characterized in this study. Subsequently, a further four *L. garvieae* isolates were selected and used for vaccination and antibody production in rabbits. The choice of these isolates was based on the cross-reactivity studies obtained with the antibodies produced by the first vaccination. To evaluate differences in cell surface antigens, an indirect enzyme-linked immunosorbent assays (ELISAs) was developed, in which all rabbit produced anti-*L. garvieae* (KM409663, KM409673, KM409680, KM409682 and KM409689) and anti-*Lactococcus* (KM409665) polyclonal antibodies were tested against the remaining *Lactococcus* isolates (n=50) as well as the two *Weissella* species and three *C. maltaromaticum* isolates.

3.2 MATERIALS AND METHODS

3.2.1 Bacterial suspension preparation

Of the 50 *Lactococcus* strains characterized in this study, six isolates were selected for the production of polyclonal antibodies in rabbits. Information on each of the selected isolates is listed in Table 3.1. The selection of isolates to be used for antibody production was based on the phylogenetic results obtained in Chapter 2. Initially, two strains were selected for antibody production. The first was *Lactococcus* isolate KM409665, which formed a sister group with *L. lactis* GQ845022 in the phylogenetic analysis, whereas the second isolate was *L. garvieae* KM409673, which clustered together with the remaining *L. garvieae* isolates. An additional four *L. garvieae* isolates were selected for subsequent antibody production, namely isolate KM409682, KM409663, KM409680 and KM409689.

To prepare bacterial suspensions for vaccination purposes, selected isolates were cultured on trypticase soy agar (TSA) and incubated at 24 ºC for 48 hrs. Following incubation, a single colony of each isolate was resuspended in phosphate buffer saline (PBS, pH 7.2) solution to achieve an OD_{660} = 0.2. Aliquots of the prepared bacterial isolates were stored at -20 ºC until needed. The same method was applied for all the isolates used in this study for cross-reactivity analysis.

Table 3.1: List of strains selected for antibody production, their date of isolation and origin.

Strains selected	**Isolation date**	**Origin**
Lactococcus isolate KM409665	13.02.2012	Farm A
L. garvieae KM409663	11.03.2010	Farm D
L. garvieae KM409673	22.10.2009	Farm B
L. garvieae KM409680	02.03.2009	Farm B
L. garvieae KM409682	07.01.2008	Farm F
L. garvieae KM409689	17.04.2009	Farm A

3.2.2 Rabbit injections and antisera isolation

Before injections were performed, blood was collected from each rabbit via the central artery of the ear, incubated for 30 min at 37 ºC and then incubated further overnight at 4 ºC. These

antisera served as negative controls. Intravenous injections were made into the marginal ear vein of each rabbit with 200 µl of the respective bacterial suspensions (prepared as described in section 3.2.1). Each rabbit was injected at the following time intervals: day 0, 11, 27 or 28, 47 or 49, 63 or 70, 104 and 129. Intravenous vaccination was employed due to the success achieved with this approach previously (Bellstedt et al., 1986; Bellstedt et al., 1987).

For isolates *Lactococcus* KM409665 and *L. garvieae* KM409673, blood was collected from each rabbit at three different time points (0, 28 and 129 days). At days 0 and 28, about 2 ml of blood was collected, whereas at day 129, a larger volume (10 ml) of blood was collected. For isolate KM409680, blood collection was made at day 0 (2 ml), day 28 (2 ml) and day 104 (10 ml). For isolate *L. garvieae* KM409663, blood was also collected at day 0, 27 and day 47 (2 ml on each occasion) and lastly on day 70 (10 ml blood). Finally, for isolates *L. garvieae* KM409689 and *L. garvieae* KM409682, blood was collected at days 0, 11 and 49 (8 ml blood on each occasion), and a final sample collected on day 63 (10 ml blood). On each occasion, blood was allowed to coagulate by incubation at 37 °C for 30 min, followed by an overnight incubation at 4 °C. The clotted blood was centrifuged at 100 × g for 20 min and the resulting supernatants (antiserum) stored at -20 ºC until needed. In this way, antibodies were produced against all 5 isolates. Ethical approval for the vaccination of all rabbits was given by the Stellenbosch University Animal Ethics Committee.

3.2.3 Antibody isolation and biotinylation

Prior to biotinylation, antibodies were isolated from the rabbit serum by ammonium sulphate precipitation. To 0.5 ml of each of the respective antisera, previously stored at -20 °C, 1.5 ml PBS (pH 7.2) and 1 ml of saturated ammonium sulfate ($(NH_4)_2$ SO_4, pH 7.2) were gently added, then incubated at 4 ºC for 20 min before being centrifuged at 15 000 × g for 20 min. After carefully removing the supernatant, the resulting pellet was re-dissolved in 1.5 ml of PBS (pH 7.2) and 1 ml saturated ammonium sulfate ($(NH_4)_2$ SO_4, pH 7.2) was added and further incubated at 4°C for 20 min. The mixture was centrifuged at 15 000 × g for 20 min and the pellet finally dissolved in 2 ml PBS (pH 7.2). The re-dissolved antibodies were dialyzed against carbonate buffer (0.05M $NaHCO_3$, pH 8.3) at 4 ºC overnight in a 10 mm x 6 mm cellulose membrane dialysis tube (Sigma-Aldrich), with one buffer change after 5 hrs of incubation. After overnight dialysis, the volume of the sample in the dialysis tube was determined and the concentration was

measured at OD_{280} on a Nanodrop ND 1000 spectrophotometer. The isolated immunoglobulin sample was diluted to a concentration of 5 mg/ml in a carbonate buffer (0.05M $NaHCO_3$, pH 8.3).

Biotinylation involves the reaction of the N-+-biotinyl-6-aminoacaproic acid-N-succinimidyl ester with the isolated antibodies to result in covalent coupling of the biotin moiety to the isolated antibodies (referred to simply as biotin). A 0.002 g of the above reagent, was dissolved in 1 ml of dimethylformamide (DMF) (2:1 ratio). The solution (biotin + DMF) was added into the 5 mg/ml antibody solution drop wise and stirred gently for 2 hrs at room temperature. The final reaction mixture was dialysed in a 25 mm wide cellulose membrane dialysis tube (Sigma-Aldrich) against 5 L of 1 × PBS (pH 7.2) at 4 ºC overnight with a single buffer change after 5 hrs of incubation. The final volume was measured and the conjugate solution was mixed with an equal volume of glycerol and stored at -20 ºC until needed.

3.2.4 Enzyme-linked immunosorbent assay (ELISA) Protocol

To assess the binding of the produced polyclonal antibodies (antigens) to each of the bacterial isolates, an ELISA was used which is shown diagrammatically in Figure 3.1. The methodology was adopted from Van Tonder (2013) but with a few modifications. Briefly, each row of each plate was coated from well 1-11 (well 12 uncoated) with 100 µl of a 1:400 dilution of antiserum in carbonate buffer (0.05 M $NaHCO_3$, pH 9.6) [28 µl serum in 11200 µl carbonate buffer] as shown in Figure 3.2. The plates were incubated at 24 °C for 2 hrs before incubation overnight (± 15 hrs) at 4 ºC after which the contents were discarded. Non-specific binding was blocked by addition of 200 µl casein buffer [154 mM NaCl, 10 mM Tris-HCl, 0.02% thiomersal and 0.5% casein (pH 7.6)] per well, including the uncoated well 12, followed by incubation for 1 hr at 37 °C after which the contents were discarded. The bacterial isolates to be tested, already suspended in 1 × PBS (pH 7.2) at OD_{660} = 0.2, were mixed separately in 1.5 ml microcentrifuge tubes with Casein-Tween 20 [Casein buffer (pH 7.6) plus 0.1% Tween 20] at a 1:10 ratio and were incubated at 37 °C for 30-60 min before use after which the contents were discarded. One hundred µl of Casein-Tween 20 was used for dilution purposes, added to wells 1 and 3-11 (serial dilutions) before adding 100 µl of the bacterial isolate suspended in Casein-Tween 20 to wells 2, 3 and 12 only. Two-fold serial dilutions were performed by pipetting 100 µl from well 3 (containing 200 µl bacteria- Casein-Tween 20) and transferring to well 4 up to well 11 (all

containing 100 µl of Casein-Tween 20), being careful to mix the suspension prior to each transfer. The plates were then incubated at 37 °C for 60 min after which the contents were discarded. Tween 20 was specifically added to the buffer to prevent any non-specific binding of the bacteria. After incubation, wells were rinsed 3 times with 300 µl of 1 × PBS-Tween 20 [PBS (pH 7.2) plus 0.1% Tween 20].

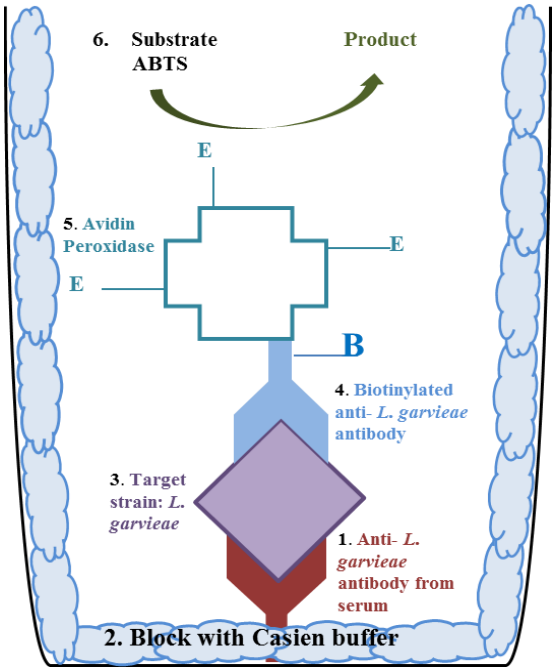

Figure 3.1: A diagram summarizing the protocol used for the sandwich ELISA performed in this study. **1**. An antibody (anti-*L. garvieae*/ anti-*Lactococcus* isolate) was coated on to the plate. **2**. Non-specific binding was blocked with casein buffer (pH 7.6). **3**. A sample to be tested (antigen) was added and if antibodies specific for the antigen were present, they would bind. **4**. A biotinylated secondary detector antibody is added that binds to the bound antigen- antibody immobilized in the well. **5**. Peroxidase-labeled avidin was added, which binds with high affinity and specificity to biotin. **6**. A substrate (ABTS), used in combination with hydrogen peroxide, was added to detect antigen- antibody complexes, for a peroxidase enzyme reaction that produces a green colored product when positive. The absorbance of the product that formed was then measured at 405 nm.

One hundred µl of a 1:100 dilution of the biotinylated antibodies (in Casein-Tween 20, kept at 37 °C for 30-60 min before use) was added to each well. After incubation for 1 hr at 37 ºC, the contents of each plate were discarded and plates were washed 3 times with 1 × PBS-Tween 20 (pH 7.2). A 100 µl aliquot of a 1:100 AvPO mix [AvPO with Casein-Tween 20 buffer (pH 9.6)] was then added to each well and plates were incubated at 37 ºC for another hour. The final step involved addition of 100 µl/well of substrate [2, 2'Azino- bis (3-ethylbenzothiozoline-6-

sulphonic acid (ABTS)] (6 mg) dissolved in in 12 ml of 0.1 M citrate buffer (pH 5) prepared 20 min before use, to which 6 µl H_2O_2 was added. ABTS and hydrogen peroxidase are used as the substrates which upon conversion by the peroxidase enzyme give a green coloured product. Plates were then incubated for 30 min at 37 °C. Plates were read on a microtitre plate reader (Biotek Instrument Inc. model Epoch) at OD_{405}. The first column of each plate served as a blank, all other components were included except for addition of bacteria.

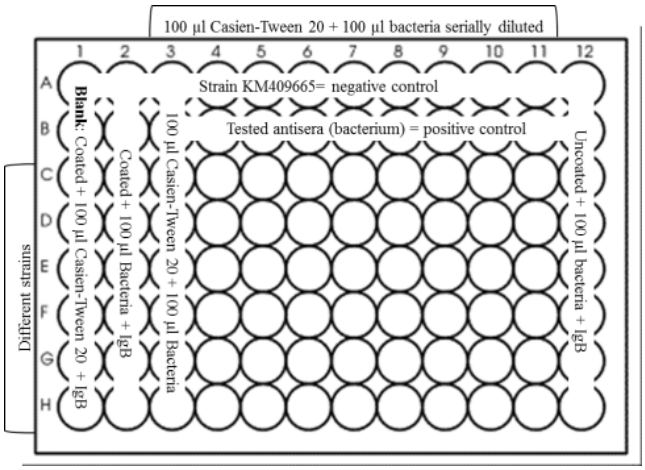

Figure 3.2: A diagram showing the 96 well plate layout used in this study. IgB = biotinylated antibodies.

3.2.5 Antibody cross reactivity determination of bacterial isolates

Each produced antibody was assessed for binding with the bacterium against which it was produced. For every ELISA performed, the bacterium against which the antibodies were produced was always included as a positive control. From all the ELISAs done for each antiserum, an average of the absorbances obtained, from the positive control, was calculated to give an overview of how each bacterium cross reacted towards its own antisera. The absorbance values obtained with the 1:100 dilution of all bacterial strains tested on the same plate was expressed as a percentage of the positive control to indicate cross reactivity. The percentage cross reactivity was divided into 6 ranges; namely 0-19, 20-49, 50-79, 80-99, <100 and <150%.

3.2.6 Cross-reactivity with other bacterial species identified in this study

Due to antigenic variation and the possibility that antigenic surfaces could be shared between species of different families, features that could assist in the pathogenicity of these bacteria, a cross reactivity assessment against the three *Carnobacterium maltaromaticum* and two *Weissella* isolates, listed in table 3.2, was performed.

Table 3.2: Strains selected for cross-reactivity against anti-*L. garvieae* antibodies

Bacterial strains	**Isolation date**	**Origin**
Weissella isolate KM409657	11.04.2011	Farm B
Weissella isolate KM409663	16.03.2011	Farm B
C. maltaromaticum KM409658	25.08.2008	Farm B
C. maltaromaticum KM409660	23.12.2009	Farm A
C. maltaromaticum KM409659	03.02.2011	Farm A

3.3 RESULTS

3.3.1 Antibody cross reactivity of bacterial isolates

The total number of isolates that fell within each of these ranges was determined to gain a better understanding of which antibody produced the best overall cross-reactivity, i.e. which bacterium would be the best candidate for developing a vaccine of high efficacy. Absorbance value less than 0.2 were regarded as non-reactivity (negative), whereas values above 0.2 were regarded as positive. This value was chosen as all control values on column 1 of each plate were less than 0.2. Also, all non-reactive values against the *Lactococcus* isolate KM409665 were less than 0.2, which would give a percentage less than 6%. The overall results obtained for antibody cross reactivity which are in percentages are displayed in table 3.3 below and again displayed on columns next to a phylogenetic tree (fig 3.3) of the *Lactococcus* isolates.

The majority of the tested strains displayed strong cross reactivity with *L. garvieae* KM409663 antibodies, with 80-99% cross reactivity (n=16) and with 12 isolates displaying cross reactivity above 100%. Conversely, 11 isolates did not cross react with *L. garvieae* KM409663 (< 19% absorbance). Low percentage cross reactivity (20-49%) was observed for the majority of

the isolates (n=33) against *L. garvieae* KM409680 antibodies, with only 2 isolates obtaining values between 50-79%, while the remaining 14 isolates did not cross-react with antibodies produced against isolate *L. garvieae* KM409680. A wide range of cross-reactivity was observed with *L. garvieae* KM409689 antibodies. About 34% (n=18) isolates had a high percentage cross reactivity above 100% and 25% (n=20) isolates had between 80- 99% reactivity. However, 45% of the isolates (n= 22) had between 50-79% reactivity, six isolates (12%) had between 20-49% and 2 isolates did not react with *L. garvieae* KM409689. These results indicate that isolate *L. garvieae* KM409689 shares most of its cell surface antigens with all the *L. garvieae* isolates in this study. As would be expected, all 49 *L. garvieae* strains had no cross reactivity against the *Lactococcus* isolate KM409665 antibodies, which is a strain closely related to *L. lactis* based on the phylogenetic analysis.

Table 3.3: Results of the total reactivity of all forty- nine tested *L. garvieae* strains against each antibody. The colours relate to their origin (**Farm A**, **Farm B**, **Farm C**, **Farm D**, **Farm E**, **Farm F** and **Farm G**)

Antibodies	**Isolation date**	**Reactivity (%)**	**Number of isolates with positive reactivity**					
			0-19	**20-49**	**50-79**	**80-99**	**<100**	**<150**
***Lactococcus* sp. KM409665**	13.02.2012	1	49 (98 %)	0	0	0	1 (2 %)	0
***L. garvieae* KM409663**	11.03.2010	38 (76 %)	11 (22 %)	1 (2 %)	9 (18 %)	16 (32 %)	11 (22 %)	1 (2%)
***L. garvieae* KM409673**	22.10.2009	41 (82 %)	9 (16 %)	4 (8 %)	6 (12 %)	13 (26 %)	17 (34 %)	0
***L. garvieae* KM409680**	02.03.2009	35 (71%)	14 (28 %)	33 (66 %)	2 (4 %)	0	0	0
***L. garvieae* KM409682**	07.01.2008	48 (96%)	1 (2 %)	2 (4 %)	4 (8 %)	10 (20 %)	26 (52 %)	6 (12%)
***L. garvieae* KM409689**	17.04.2009	48 (96%)	1 (2 %)	3(6 %)	7 (14 %)	20 (40 %)	16 (32 %)	2 (4%)

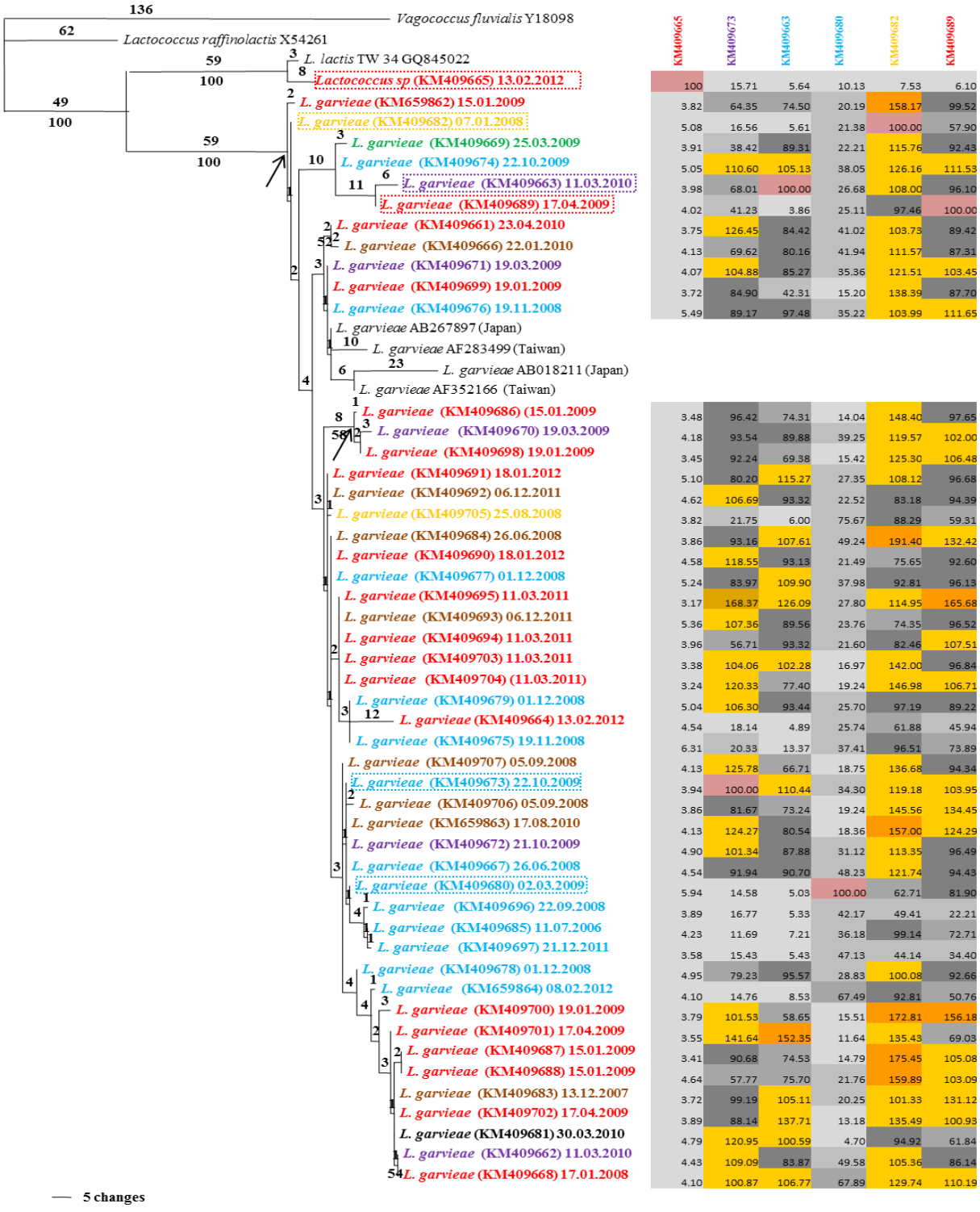

Figure 3.3 A phylogenetic tree derived from parsimony analysis of the 16S rRNA gene sequence of forty-nine *L. garvieae* and one *Lactococcus* isolates. Sandwich Enzyme-linked Immunosorbent Assay (ELISA) result obtained for all six sera produced against: *Lactococcus* sp KM409665; KM409673; KM409663; KM409680; KM409682 and KM409689. The colours relate to their origin (**Farm A**, **Farm B**, **Farm C**, **Farm D**, **Farm E**, **Farm F** and **Farm G**). The numbers are percentages of reactivity and coloured boxes relate to the intensity of reactivity: <20%; 20-49%; 50-79%; 80-90%; 100-149% and >150%.

3.3.3 Cross reactivity with other species identified within this study

No cross reactivity occurred between the three *C. maltaromaticum* isolates (KM409663, KM409689 and KM409665) and all the antibodies tested in this study. No cross reactivity was observed between the two *Weissella* isolates (KM409657 and KM409656) using antibodies against *Lactococcus* isolate KM409665, but a slight reactivity was noted with antibodies produced against *L. garvieae* KM409680 for both *Weissella* isolates. Low cross-reactivity was also noted for *Weissella* isolate KM409656 when using anti-*L. garvieae* KM409682 antibodies. This result is interesting in that very low levels of cross reactivity were observed for all *L. garvieae* isolates when using anti-*L. garvieae* KM409680 antibodies. These findings may be indicative of similar surface antigens shared between these different genera, which may be representative of proteins that are common to these bacteria such as typical ABC transmembrane transporters (Mogensen, 2009).

Table 3.4: Cross reactivity of other non-*Lactococcus* isolates against the 6 anti-*Lactococcus* antibodies. The colours relate to their origin (**Farm A**, **Farm B**, **Farm C**, **Farm D**, **Farm E**, **Farm F** and **Farm G**)

	Weissella sp. KM409657 (11.04.2011)	*Weissella* sp. KM409656 (16032011)	*C. maltaromaticum* KM409658 (25.08.2008)	*C. maltaromaticum* KM409660 (23.12.2009)	*C. maltaromaticum* KM409659 (03.02.2011)
Antibodies	**Reactivity percentage**				
***5 Lactococcus* sp. KM409665**	3.37	3.63	3.53	3.40	3.22
***14 L. garvieae* KM409673**	3.90	4.19	3.06	3.09	2.95
***3 L. garvieae* KM409663**	7.61	7.13	3.76	3.18	3.58
***24 L. garvieae* KM409680**	42.65	31.56	10.11	9.50	9.38
***26 L. garvieae* KM409682**	15.89	23.63	8.46	8.57	8.46
***34 L. garvieae* KM409689**	7.52	9.25	4.41	4.60	4.41

3.4 DISCUSSION

There are several factors that contribute towards bacterial pathogenicity which need to be considered when assessing pathogenicity traits amongst different strains of the same species (intraspecies). Numerous studies have looked at the diversity of *L. garvieae* strains isolated from diseased rainbow trout farmed from different geographical regions and have compared the isolates in terms of their genotype, phenotype and serological characteristics. A few of these studies concluded that there is great phenotypic heterogeneity among *L. garvieae* isolates from different hosts and origin or geographic locations (Liébana et al., 2000; Eldar et al., 1999), whereas a few suggest that there are high levels of similarities or phenotypic homogeneity among these *L. garvieae* isolates even within those of the same geographic location (Altun et al., 2007; Altun et al., 2013; Eldar et al., 1999).

Based on the results obtained from this study, there appear to be significant antigenicity differences/diversity amongst the *L. garvieae* strains isolated from both Lesotho and South Africa. It appears as though the origin and the year of isolation makes no significant difference to the reactivity portrayed. For instance, isolates *L. garvieae* KM659862, *L. garvieae* KM409686, *L. garvieae* KM409687 and *L. garvieae* KM409688 were isolated from Farm A on the same date (15.01.2009), but displayed a different pattern of cross reactivity. Similar findings were recorded for many of the other isolates analyzed in this study. There could be several reasons for this pattern of cross reactivity. Foley et al. (2009) explains that in bacteria, expressed antigen variants tend to vary genetically over the course of infection, often due to adaptations to host immunity. Unlike in viruses, mechanisms of antigenic variation in most bacteria involve larger DNA movement, such as gene conversion or DNA rearrangement, although some antigens vary due to point mutations or modified transcriptional regulation. Other mechanisms include mutations underlying variant production; drift which could remove alleles especially early in infection (Foley, 2009).

L. garvieae KM409680 showed very low levels of surface antigens with 66% (n=33) of the isolates cross reacting with its antisera with reactivity percentages of between 20-49%. Based on these results, *L. garvieae* KM409680 would not be the best candidate to be selected for vaccine development since the cross reactivity is very low. Both isolates *L. garvieae* KM409682 and *L. garvieae* KM409689 antibodies cross reacted strongly at 98% cross reactivity, respectively at

52% and 32% cross reactivity above 100%. Therefore, these two isolates (*L. garvieae* KM409680 and *L. garvieae* KM409 689) would be the best candidates for vaccine development. In addition, isolates *L. garvieae* KM409673 and *L. garvieae* KM409663 also appear to be good candidates for vaccine development with 82% and 76% cross reactivity observed respectively, with the highest number of isolates (34%) sharing their cell surface above 100% with isolate *L. garvieae* KM409673. The *Lactococcus* isolate KM409665 proved itself to be different again when none of the *L. garvieae* isolates, including the two *Weissella* isolates and the three *C. maltaromaticum* isolates, did not cross react with the antibodies produced against it. This isolate appears to not share any of its cell surface antigens with any of the bacteria characterized in this study.

CHAPTER 4: CONCLUSIONS AND FUTURE PERSPECTIVES

Vaccination is one of the best ways of controlling bacterial diseases, and has been routinely used in aquaculture to successfully prevent outbreaks of bacterial diseases in a number of fish species (Bravo and Midtlyng, 2007; Alvarez-Pellitero, 2008). Correct characterization and identification of pathogenic bacteria is, however, an essential prerequisite for determining treatment options and for the development of effective vaccines. This is particularly important for diseases such as streptococcosis or lactococcosis, which are caused by a number of Gram-positive bacteria and where a high degree of phenotypic heterogeneity exists amongst isolates of the same species (Gomes et al., 2006; Klesius et al., 2006; Erfanmanesh et al., 2012).

The findings of this study are in agreement with the current literature regarding the morphological characteristics of *Lactococcus*, *Weissella* and *Carnobacterium*, which were all characterized in this study to be Gram positive, non-motile, catalase and oxidase negative bacteria. It was not possible to identify the *L. garvieae* isolates using the API 50CH system, but it was possible to show that there was some intraspecies phenotypic heterogeneity between the *L. garvieae* isolates using the API 50CH system and the growth characteristics. However, Gibello et al. (2016) suggests the use of API 32 strep instead of API 50CH system for the characterization of *L. garvieae* strains. This is in contradiction to Fukushima et al. (2017), who found results from API 32 strep to be inconclusive for the identification of *L. garvieae* BR-LG3 used in their study. It would therefore be sensible to re-evaluate the isolates included in this study using the API 32 Strep Kit. Even though the isolates in this study were shown to be non-motile, some *L. garvieae* isolates from lactococcosis cases had been identified to be in possession of locomotive structures. Ooyama et al. (2002) was the first to observe pili or fimbriae in *L. garvieae* strains isolated from yellowtail (*Seriola lalandii*) with lactococcosis. The same observation was made by Gibello et al. (2016) of several *L. garvieae* strains where comparative genomic analysis revealed the existence of a pili gene cluster with a high genetic diversity among the different isolates that were studied. Whether some of the isolates tested in the present study possess a pilli gene and determining under which circumstances these pilli are expressed is information worth investigating.

A new species of *Weissella* was identified in this study, isolated from rainbow trout farmed in South Africa and exhibiting clinical signs of weisselliosis. The two strains are genotypically

homogenous to the Brazilian, United States and Chinese isolates as shown through phylogenetic analysis in chapter 2. However, the physiological assessment of the two *Weissella* isolates characterized in this study is not in agreement with the rest of the *Weissella* isolates characterized from China, Brazil and the United States. Growth is reported to occur between 20-45 °C (Buller, 2014), whereas these two isolates could not grow at 45 °C. However, such findings can be expected as isolates of the same species can differ in their physiology. Furthermore, both isolates could not be identified through the API 50CH kit on the basis of their carbon source assimilation. Further characterization of these two isolates to a species level is still to be conducted.

One of the *Lactococcus* species identified in this study, *Lactococcus* isolate KM409665, was shown to be genotypically similar to *L. lactis* GQ845022. This isolate (*Lactococcus* KM409665) was phenotypically indistinguishable from all the other *L. garvieae* isolates based on its growth on TSA and BA by having the same colony morphology. However, the isolate was found to be biochemically different from all the other isolates tested in this study based on the API 50CH system and was clearly identified as *L. lactis*. This isolate also differed physiologically from the other *L. garvieae* isolates in that it grew very well at 10 ºC. Further characterization to further distinguish this isolate still needs to be performed to determine whether this isolate is a new species.

C. maltaromaticum was also identified in this study. The three isolates were similar to three other non-pathogenic *C. maltaromaticum* isolates: 1) *C. maltaromaticum* B26 (AM179873), which is part of the normal intestinal flora of trout (Kim & Austin, 2008); 2) *C. maltaromaticum* MMF14 (GQ304922) obtained from a chinook salmon with no clinical signs of disease (Loch et al., 2011); and 3) *C. maltaromaticum* MF204 (AY543031) isolated from modified-atmosphere packaged (MAP) salmon (Rudi et al., 2004). The isolates identified in this study were isolated in three separate cases from diseased rainbow trout obtained from Lesotho [*C. maltaromaticum* KM409660 (23.12.2009), *C. maltaromaticum* KM409659 (03.02.2011)] and South Africa [*C. maltaromaticum* KM409658 (25.08.2008)]. How pathogenic these isolates are to rainbow trout needs to be further investigated through fish infection trials.

The data presented in this study supports the use of genetic data for more definitive species identification. The phylogenetic analysis conducted in Chapter 2, indicates that *L. garvieae* is the

dominant species infecting rainbow trout farms in South Africa and Lesotho. Through PCR, sequencing and phylogenetic analysis of the 16S rRNA gene, the isolates grouped with other *L. garvieae* strains isolated from lactococcosis cases. About 89% (n=49) of the bacterial isolates were identified as *L. garvieae*. Similar findings were reported by Bekker et al. (2011), where out of the twelve bacterial isolates previously isolated by Bragg & Broere (1986) from Streptococcosis outbreaks in rainbow trout from South Africa, 58% (n=7) of the isolates were identified as *L. garvieae*. From these findings, it is therefore concluded that *L. garvieae* remains to be one of the main causative agents of lactococcosis on rainbow trout farms in South Africa following its initial diagnosis around 1986.

From the antigenicity studies performed in chapter 3, the results indicated that there was significant diversity amongst the *L. garvieae* strains isolated from diseased rainbow trout farmed in Lesotho and South Africa. During 2006-2012, the vaccines produced from some of these strains could not be used repeatedly over the years, which was due to their serological differences which were never properly assessed. This study was able to show that these *L. garvieae* strains differed in their cell surface antigens regardless of the origin and date of isolation (as some were isolated on the same dates). As much as PCR and sequencing were valuable in identifying and differentiating these isolates at a species level, they did not provide adequate information about the different serotypes or surface antigens that may exist among them, which is critical information in making final conclusions about the strains to be considered for development of vaccines with good efficacy for treatment of lactococcosis on these farms. From this study, it can therefore be concluded that more than one serotype of *L. garvieae* exists within this group of *L. garvieae* isolates. *L. garvieae* KM409682 was found to be the best candidate for vaccine development, as it shared the majority of its surface antigens with most of other isolates analyzed in this study. To a very slight extent, there might be shared antigenicity that exists between two *L. garvieae* isolates (KM409682 and KM409680) and the two *Weissella* isolates, and to a very slight extent to the three *C. maltaromaticum* isolates. This could be indicative of similar virulence factors which may be transferred through possible horizontal gene transfer between isolates that would exist in one environment in close proximity, but further investigation to confirm this would be necessary. Whether any of the isolates possesses a capsule remains to be determined, but this information may assist in explaining the differences noted in this study.

In future, this study can be expanded through vaccine development from some of the strains characterized. The aim would be to prepare whole cell vaccines and DNA vaccines, and to perform vaccinations and infection trials to evaluate which type of vaccine would be more effective in reducing infections. Fish infection trials should also be performed to record the infection rate, infection routes and disease symptoms caused by each of the different species: *L. garvieae*, *Lactococcus* isolate, *Weissella* isolates, and *C. maltaromaticum*. Further investigations could also be performed on virulence factors harbored by these species using different methods: isolation of plasmids and capsules, and detection of virulence related genes such as the capsule gene cluster, plasmid isolation and any toxins that may be of importance for pathogenicity of these isolates in rainbow trout.

Virulence factors of *L. garvieae* isolates from lactococcosis cases have recently been studied. Ture & Altinok (2016) studied the existence of putative virulence genes in *L. garvieae* isolates from rainbow trout in Turkey, France, Iran, Italy, and Spain and from yellowtail (*Seriola quinqueradiata*) in Japan. The study assessed the presence of several genes including a capsule gene cluster (CGC), hemolysins, NADH oxidase, phosphoglucomutase, adhesin, adhesin clusters, adhesin Pav, adhesin PsaA, enolase, superoxide dismutase and LPxTG-(Leu-Pro-any-Thr-Gly) containing surface proteins. Their study revealed that a capsule is not essential for virulence of *L. garvieae* and it might be a combination of different genes that is responsible for the virulence of *L. garvieae*. Several studies have demonstrated the protein glyceraldehyde-3-phosphate dehydrogenase (GPDH) to be a good candidate for vaccine development against lactococcosis and streptococcosis in rainbow trout and Nile tilapia, based on its ability to induce an immune response by production of protective antibodies (Tsai et al., 2013; Zhang et al., 2017; Trung Cao et al., 2014). The prospect of using this protein as a potential candidate for vaccine development against *L. garvieae* infections in rainbow trout farmed in South Africa and Lesotho has not been investigated yet and needs to be considered. The existence of some of these virulence genes, basically the capsule gene cluster, would be an important consideration in an effort to study the virulence components of the *L. garvieae* strains affecting rainbow trout in South Africa and Lesotho.

Another part of research that needs to be further investigated is the difference, if any, in efficacy between polyclonal antibodies produced by rainbow trout and those we have produced

from rabbits. This would help us note/ understand if the six produced antibodies would have the same binding affinity as those produced from rainbow trout. This study would give an insight in response differences between mammals and fish and allow us to note the different responses that a mammal may have as opposed to a fish. In future studies, information needs to be gained in: 1) knowing which pathogenicity traits are involved in rainbow trout infections by these isolates; 2) selecting which genes would be necessary for consideration for production of DNA vaccines; 3) to clarify the specific roles of these proteins that have not yet been described in the virulence of *L. garvieae*, and which may help to understand their strategies when infecting rainbow trout.

This study has been particularly valuable in the identification and characterization of bacterial isolates in Lesotho and South Africa, and which can be used as vaccine candidates. This study has improved the knowledge of the genetic diversity of the bacterial species isolated from diseased farmed trout in Lesotho and South Africa that would facilitate development of vaccines that could cover all strains and/or isotypes and may ultimately improve the efficacy and potency of the produced vaccines. This can have a significant impact on the economic viability of rainbow trout farming in Lesotho and South Africa.

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APPENDIX A (Media, Solutions and Protocols)

Chapter 2: General stock solutions

Table A.1: General stock solutions of buffers and chemicals used for in this study.

Solution	Method
3 % Hydrogen peroxide	3 ml in 10 ml dH_2O
1 M Tris-Cl (100 ml)	Tris base 12.1 g, ddH_2O 70 ml, HCL 6 ml
0.5 M EDTA (50 ml)	EDTA 9.305 g, NaOH 1 g. Dissolve the EDTA and NaOH in 40 ml of dH_2O, adjust the pH to 8.0 and make up to a final volume of 50 ml.
Tris-EDTA buffer (TE) pH 7.6 (100 ml)	1 M Tris-Cl (pH 7.6) 1 ml, 0.5 M EDTA 200 µl, ddH_2O 98.8 ml
DNA Marker (1-10 kb)	Mix 5 µl of DNA marker with 1 µl of 6X bromophenol blue (loading dye), Working stock: 150 µl DNA marker + 30 µl loading dye.
50 X TAE (Tris-Acetate-EDTA buffer)	Tris-base 242 g, 0.5 M EDTA 100 ml, Glacial acetic acid 57.1 ml dH_2O 1 L
1 X TAE buffer	Tris-base 4.84 g, 0.5 M EDTA 2 ml or 0.744 g, Glacial acetic acid 1.142 ml, dH_2O 1 L
Ethidium bromide 10 mg/ml	Dissolve 0.1 g in 10 ml dH_2O, Use 3 µl in 100 ml Agarose gel.
Gel tracking dye (6 X)	Bromophenol blue 62.5 g, Sucrose 10.0 g, 0.5 M EDTA 1 ml, dH_2O 25 ml [Do not autoclave].
1 % Agarose gel	Agarose 1 g, 1X Tris-acetate buffer 100 ml
10 X Phosphate Buffered Saline (PBS)	NaCl 80 g , KCl 2 g , Na_2PO_4 (Merck) 14.4 g , KH_2PO_4 (Saarchem) 2.4 g , dH_2O to 1 L . Dissolve NaCl, KCl, Na_2PO_4 and KH_2PO_4 in 900 ml of dH_2O, adjust the pH to 7.4 and make up to 1 L with dH_2O.
1 x PBS	Dilute 10 x PBS 1:10 with dH_2O
1 M $CaCl_2$	$CaCl_2.2H_2O$ 14.7 g , dH_2O to 100 ml

Table A.2: List of antibiotics used in this study and their preparation methodology.

Antibiotic group	Members	Primary effect	Mode of action	Spectrum
Cell Wall Synthesis Inhibition				
Penicillins	**Penicillin G** (10 µg) (Raissy & Ansari, 2011) Dissolve 150 mg in 15 ml of deionized water. Sterilize in 0.2 µm filter syringe and store aliquots at -20 ºC.	Cidal	Cell wall inhibition (Peptidoglycan synthesis)	Narrow (Gram positive)
Glycopeptides	**Vancomycin** (30 µg)			Aerobic & anaerobic Gram positive bacteria.
Cephalosporins	**Cefotaxime** (10 µg) (Raissy & Ansari, 2011) Dissolve 150 mg in 15 ml deionized water, filter sterilize in 0.2 µm syringe filter and store aliquots at -20 ºC.			Broad (Gram positive and some Gram negative)
Protein Synthesis Inhibition				
Macrolides	**Erythromycin** (15 µg) (Liu et al, 2009) Dissolve 225 mg in 95% ethanol and filter sterilize in 0.2 µm syringe filter and store aliquots at -20 ºC.	Static	Protein synthesis	Broad (Aerobic & anaerobic Gram positive & Gram negative)
Aminoglycosides	**Gentamicin (10 µg)** Dissolve 150 mg in 15 ml deionized water, then filter sterilize in 0.2 µm syringe filter and store aliquots at -20 ºC. **Streptomycin(10 µg)** Dissolve 750 g in 15 ml of deionized water. Filter	Cidal	Protein synthesis	Broad (Gram negative, mycobacteria)

	sterilize in 0.2 µm syringe filter and store aliquots at -20 ºC. **Kanamycin (30 µg)** Dissolve 750 mg in 20 ml of deionized water. Filter sterilize in 0.2 µm syringe filter and store aliquots at -20 ºC. (Liu et al, 2009; Raissy & Ansuri, 2011)			
Tetracyclins	**Oxytetracycline (30 µg)** Dissolve 450 mg in	Static	Protein synthesis inhibition	Broad (Gram positive, Gram negative)
Phenicols	**Chloramphenicol (30 µg)** Dissolve 510 g in 15 ml of ethanol. Filter Sterilize in 0.2 µm syringe filter and store aliquots at -20 ºC.	Staric	Protein synthesis inhibition	Broad (Gram positive, Gram negative)
Nucleic acid synthesis inhibition				
Quinolones and fluoroquinolones	**Norfloxacin (10 µg)** Dissolve 150 mg in 14. 850 ml (14 ml, 850 µl) deionized water, mix with 150 µl acetic acid.	Cidal	Nucleic acid synthesis inhibition (inhibits the bacterial DNA gyrase or the topoisomerase IV enzyme)	Narrow (Gram negatives better than Gram positives)
Antimetabolites				
Sulfonamides	**Sulphamethoxazole (25 µg)** (Liu et al, 2009; Raissy & Ansuri, 2011)	Static	Antimetabolites: inhibits folic acid synthesis by competing	Broad spectrum

	Dissolve 375 mg in 7.5 ml deionized water, mix with 0.1 mol/L NaOH (0.06 g NaOH in 15 ml deionized water) to dissolve.		with PABA	

Chapter 3: ELISA Buffers and Protocol

Table A.3: List of buffers and reagents used for ELISA.

Phosphate buffer pH 7.2	**10X (1L)**	**5X**	**1X (1L)**	**1X (3L)**
NaCl	80 g	40 g	8 g	24 g
KCl	2 g	1 g	0.2 g	0.6 g
$Na_2HPO_4 \cdot 2H_2O$	14.24 g	7.12 g	1.424 g	4.272 g
KH_2PO_4	2 g	1 g	0.2 g	0.6 g
0.1% tween 20			100µl	300µl

Carbonate buffer pH 9.6	**1L**	**250ml**	**1:20 dilution (100ml)**
Na_2CO_3	1.6 g	0.4 g	5 ml + 95 ml dH_2O
$NaHCO_3$	2.9 g	0.725 g	
Working stock use 1:20 dilution			

Citrate buffer pH5	**200ml**
Citric acid $C_6H_8O_7$	1.47 g
Trisodium citrate $Na_3C_6H_5O_7$	3.824 g

Casein buffer pH 7.6	**500ml**	**1L**
154mM NaCl	4.50 g	9 g
10mM Tris/HCl	0.788 g	1.576 g
0.02% Thiomersol	0.1 g	0.2 g
0.5% Casein	2.5 g	5 g
Balance pH first before adding casein, leave on stirrer overnight		

Table A.4: ELISA protocol.

STEPS	PROCEDURE	AFTER INCUBATION
Coat 1:400	28 µl serum + 11 200 µl carb. pH9.6 100 µl wells 1-11 uncoated 12 Incubate @ 4 °C overnight	FLICK
Block	Casein buffer pH7.6 without tween 200 µl/well including 12 Incubate @37°C 1hour	Wash 3X PBS-tween pH7.2
Bacteria 1:10	Add 100 µl cas-twn wells 1,3—11 150 µl bacteria + 1 350 µl cas-twn (keep mix @ 37°C for 30 min-1hour) 100µl wells 2,12, 3-----$^{(\text{dilution= 100µl 6X})}$---11 Incubate @ 37 °C 1hour	Wash 3X PBS-tween pH7.2
Ig Biotin 1:100	120 µl IgB + 11 880 µl ca-twn (keep mix @ 37°C for 30 min-1hour) 100 µl/well Incubate @37 °C 1hour	Wash 3X PBS-tween pH7.2
AvPO 1:100	120 µl AvPO + 11 880 µl cas-twn 100 µl/well Incubate @ 37°C 1hour	Wash 3X PBS-tween pH7.2
Substrate	(Prepare 20 min before incubation time ends) Weigh 0.006 g ABTS + 12 ml citrate buffer pH 5 *keep citrate buffer in dark *keep mix in dark Before dispensing into plate: Add 6 µl H_2O_2 on mix 100 µl/well Incubate @ 37°C 30 min	Take readings Absorbance mode Filter 405 Blank column 1 Continuous movement

APPENDIX B (Tables and Figures)

Table B. 1: List of diseases caused by Gram positive bacteria that cause disease in rainbow trout (Buller 2014, Page 38- 40).

Host	**Pathogen**	**Tissue site**	**Disease status**
Rainbow trout (*Oncorhynchus mykiss* Walbaum 1792)	*Carnobacterium* (*piscicola*) *maltaromaticum* (rod)	Bilateral exophthalmia, periocular haemorrhages, ascites fluid and haemorrhages in liver, swimbladder, muscle and intestine, inflammation in the brain, kidney	Chronic disease with low level mortality or highly virulent.
	Corynebacterium aquaticum (rod)	Brain	Exophthalmia in experimental infection
	Clostridium botulinum (rod)	Toxin in serum and intestinal contents	Botulism
	Lactococcus garvieae (cocci)		Mortality
	Lactococcus lactis spp. *Tructae* (cocci)	Intestinal mucus	Normal flora
	Lactococcus piscium (cocci)		Pseudokidney disease, lactobacillosis
	Micrococcus luteus	Organism isolated from kidney, spleen and ascites fluid	Vibriosis, septicaemia Isolated from moribund fish
	Nocardia asteroides (rod)		Nocardiosis
	Renibacterium salmoninarum	White nodules in kidney	Bacterial kidney disease
	Staphylococcus warneri	Exophthalmia, ascites fluid, lesions on fins, organism in kidney and liver	Diseased and dying trout –opportunistic infection
	Streptococcus agalactiae	Organisms in brain and eye	Meningoencephalitis, septicaemia.
	Streptococcus iniae	Organisms in brain and eye	Meningoencephalitis, septicaemia.
	Vagococcus salmoninarum (rod)	Loss of equilibrium, haemorrhage around eye and gill, lesions on body, congestion in spleen and liver. Peritonitis, haemorrhagic ascites, retained eggs, engorged testes, languid swimming.	Vagococcosis, chronic disease with mortality.
	Weissella species	Brain, kidney, liver	Haemorrhagic septicamia

Table B. 2: List of isolates used in the construction of phylogenetic trees.

***Weissella* species and outgroups**	
***Weissella* sp. NC KC461925**	Welch, T.J., and Good, C.M., (2013). Mortality associated with Weissellosis (*Weissella* sp.) in USA farmed rainbow trout: Potential for control by vaccination. Aquaculture. 388-391, 122-127.
***Weissella* sp. HZ-K EU869289**	Liu, J.Y., Li, A.H., Ji, C., and Yang, W.M., (2009). First description of a novel *Weissella* species as an opportunistic pathogen for rainbow trout (*Oncorhynchus mykiss*) (Walbaum) in China. Veterinary Microbiology. 136 (3-4), 314-320.
***Weissella* sp. RT-1L EU869292**	Liu, J.Y., Li, A.H., Ji, C., and Yang, W.M., (2009). First description of a novel *Weissella* species as an opportunistic pathogen for rainbow trout (*Oncorhynchus mykiss*) (Walbaum) in China. Veterinary Microbiology. 136 (3-4), 314-320.
***Weissella* sp. WS-72** GU112513	Figueiredo, H.C., Costa, F.A., Leal, C.A., Carvalho-Castro, G.A., and Leite, R.C., (2012). *Weissella* sp. outbreaks in commercial rainbow trout (*Oncorhynchus mykiss*) farms in Brazil. Veterinary Microbiology. 156 (3-4), 359-366.
***Weissella* sp. WS-71 GU112512**	Figueiredo, H.C., Costa, F.A., Leal, C.A., Carvalho-Castro, G.A. and Leite, R.C., (2012). *Weissella* sp. outbreaks in commercial rainbow trout (*Oncorhynchus mykiss*) farms in Brazil. Veterinary Microbiology. 156 (3-4), 359-366.
***Weissella* sp. WS-30** GU112508	Figueiredo, H.C., Costa, F.A., Leal, C.A., Carvalho-Castro, G.A. and Leite, R.C., (2012). *Weissella* sp. outbreaks in commercial rainbow trout (*Oncorhynchus mykiss*) farms in Brazil. Veterinary Microbiology. 156 (3-4), 359-366.
***Weissella* sp. WS-14** GU112507	Figueiredo, H.C., Costa, F.A., Leal, C.A., Carvalho-Castro, G.A. and Leite, R.C. (2012). *Weissella* sp. outbreaks in commercial rainbow trout (*Oncorhynchus mykiss*) farms in Brazil. Microbiology. 156 (3-4), 359-366.
***Weissella* sp. WS-06** GU112506	Figueiredo, H.C., Costa, F.A., Leal, C.A., Carvalho-Castro, G.A. and Leite, R.C. (2012). *Weissella* sp. outbreaks in commercial rainbow trout (*Oncorhynchus mykiss*) farms in Brazil. Microbiology. 156 (3-4), 359-366.
***Weissella* sp. WS-32** GU112509	Figueiredo, H.C., Costa, F.A., Leal, C.A., Carvalho-Castro, G.A., and Leite, R.C. (2012). *Weissella* sp. outbreaks in commercial rainbow trout (*Oncorhynchus mykiss*) farms in Brazil. Veterinary Microbiology. 156 (3-4), 359-366.
***Weissella* sp. WS-08** GU112514	Figueiredo, H.C., Costa, F.A., Leal, C.A., Carvalho-Castro, G.A., and Leite, R.C. (2012). *Weissella* sp. outbreaks in commercial rainbow trout (*Oncorhynchus mykiss*) farms in Brazil. Veterinary Microbiology. 156 (3-4), 359-366.
***Weissella* sp. WS-56** GU112510	Figueiredo, H.C., Costa, F.A., Leal, C.A., Carvalho-Castro, G.A., and Leite, R.C. (2012). *Weissella* sp. outbreaks in commercial rainbow trout (*Oncorhynchus mykiss*) farms in Brazil. Veterinary Microbiology. 156 (3-4), 359-366.
***Weissella* sp. WS-65** GU112511	Figueiredo, H.C., Costa, F.A., Leal, C.A., Carvalho-Castro, G.A. and Leite, R.C. (2012). *Weissella* sp. outbreaks in commercial rainbow trout (*Oncorhynchus mykiss*) farms in Brazil. Veterinary Microbiology. 156 (3-4), 359-366.
***Weissella ceti* 1119-1A-09** NR_117039	Vela, A.I., Fernandez, A., de Quiros, Y.B., Herraez, P., Dominguez, L., and Fernandez-Garayzabal, J.F. (2011). *Weissella ceti* sp. nov., isolated from beaked whales (*Mesoplodon bidens*). International Journal of Systematic and Evolutionary. Microbiology. 61 (PT 11), 2758-2762.
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Weissella kimchii AF312874	Choi, H.J., Cheigh, C.I., Kim, S.B., Lee, J.C., Lee, D.W., Choi, S.W., Park, J.M., and Pyun, Y.R. (2002). *Weissella kimchii* sp. nov., a novel lactic acid

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***Weissella viridescens* NRIC 1536** NR_040813	Suzuki, M., and Suzuki, K. (2000). *Weissella viridescens* gene for 16S rRNA, strain: NRIC 1536. Published Only in Database.
***Vagococcus fluvialis* SS1339** GQ337040	Shewmaker, P.L., Steigerwalt, A.G., Nicholson, A.C., Carvalho Mda, G., Facklam, R.R., Whitney, A.M., Teixeira, L.M. (2011). Reevaluation of the Taxonomic Status of Recently Described Species of Enterococcus: Evidence that *E. thailandicus* Is a Senior Subjective Synonym of *'E. sanguinicola'* and Confirmation of *E. caccae* as a Species Distinct from *E. silesiacus*. Journal of Clinical Microbiology. 49 (7), 2676-2679.
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***Lactococcus lactis* subsp. *lactis* MJK16 AB494727.1**	Choi, H., Kim, S., Han, N., and Yoon, H. (Unpublished). Microbial Profile Analysis of Lactic Acid Bacteria in Various Fermented Foods.
***Lactococcus lactis* FT697** KM207824	Tulini, F.L., Hymery, N., Haertle, T., Le Blay, G. and De Martinis, E.C.P. (Unpublished). Screening for antimicrobial and proteolytic activities of lactic acid bacteria isolated from cow, buffalo and goat milk and cheese in southeast region of Brazil.
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***Lactococcus garvieae* FLG2** AF352166	Chen, S.C., Liaw, L.L., Su, H.Y., Ko,S.C., Wu, C.Y., Chaung, H.C., Tsai, Y.H., Yang, K.L., Chen, Y.C., Chen, T.H., Lin, G.R., Cheng, S.Y., Lin, Y.D., Lee, J.L., Lai, C.C., Weng, Y.J. Chu, S.Y. (2002). *Lactococcus garvieae*, a cause of disease in grey mullet, *Mugil cephalus* L., in Taiwan. Journal of Fish Diseases. 25, 727-732.
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***Lactococcus raffinolactis* NCDO 617** NR_118904	Collins, M.D., Ash, C., Farrow, J.A., Wallbanks, S., Williams, A.M. (1989). 16S ribosomal ribonucleic acid sequence analyses of lactococci and related taxa. Description of *Vagococcus fluvialis* gen. nov., sp. Nov. Journal of Applied Bacteriology. 67 (4), 453-460.
***Lactococcus garvieae* 10757/20** KM409661	Kutu, V., Bellstedt, D., Macey, B., Mouton, A. Biochemical and genetic characterization of bacteria isolated from diseased rainbow trout (*Oncorhynchus mykiss*) farmed in South Africa and Lesotho. Unpublished
***Lactococcus garvieae* JB375381/5** KM409691	Kutu, V., Bellstedt, D., Macey, B., Mouton, A. Biochemical and genetic characterization of bacteria isolated from diseased rainbow trout (*Oncorhynchus mykiss*) farmed in South Africa and Lesotho. Unpublished
***Carnobacterium* species and outgroups**	
***Carnobacterium maltaromaticum* MMF-21** GQ304929	Loch, T.P., Kumar, R., Xu, W., and Faisal, M. (2011). *Carnobacterium maltaromaticum* infections in feral *Oncorhynchus* spp. (Family Salmonidae) in Michigan. Journal of Microbiology. 49 (5), 703-713.
Carnobacterium maltaromaticum	Loch, T.P., Kumar, R., Xu, W., and Faisal, M. (2011). *Carnobacterium*

MMF-19 GQ304927	*maltaromaticum* infections in feral *Oncorhynchus* spp. (Family Salmonidae) in Michigan. Journal of Microbiology. 49 (5), 703-713.
***Carnobacterium maltaromaticum* MMF-16** GQ304924	Loch, T.P., Kumar, R., Xu, W., and Faisal, M. (2011). *Carnobacterium maltaromaticum* infections in feral *Oncorhynchus* spp. (Family Salmonidae) in Michigan. Journal Microbiology. 49 (5), 703-713.
***Carnobacterium maltaromaticum* MMF-12** GQ304920	Loch, T.P., Kumar, R., Xu, W. and Faisal, M. (2011). *Carnobacterium maltaromaticum* infections in feral *Oncorhynchus* spp. (Family Salmonidae) in Michigan. Journal of Microbiology. 49 (5), 703-713.
***Carnobacterium maltaromaticum* MMF-17** GQ304925	Loch, T.P., Kumar, R., Xu, W., and Faisal, M. (2011). *Carnobacterium maltaromaticum* infections in feral *Oncorhynchus* spp. (Family Salmonidae) in Michigan. Journal Microbiology. 49 (5), 703-713.
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***Carnobacterium divergens* B33** AM179875	Kim, D.H., Brunt, J. and Austin, B. (2007). Microbial diversity of intestinal contents and mucus in rainbow trout (*Oncorhynchus mykiss*). Journal of Applied Microbiology. 102 (6), 1654-1664.
***Carnobacterium maltaromaticum* B26** AM179873	Kim, D.H., Brunt, J., and Austin, B. (2007). Microbial diversity of intestinal contents and mucus in rainbow trout (*Oncorhynchus mykiss*). Journal Of Applied Microbiology. 102 (6), 1654-1664
***Carnobacterium divergens* MF 109** AY543016	Rudi, K., Maugesten, T., Hannevik, S.E., and Nissen, H. (2004). Explorative multivariate analyses of 16S rRNA gene data from microbial communities in modified-atmosphere-packed salmon and coalfish. Appl. Environ. Microbiol. 70 (8), 5010-5018.

Statistical Analysis Results: Temperature

Table B. 3: Descriptive statistics of the South African *L. garvieae* isolates at different temperatures.

Descriptive Statistics_ Temperature (*L. garvieae*_ South African isolates)											
Level	N	**10 °C**					**24 °C**				
Factor		Mean	Std.Dev.	Std.Err	-95,00%	95,00%	Mean	Std.Dev.	Std.Err	-95,00%	95,00%
Total	33	0,12061	0,08455	0,01472	0,09063	0,15059	1,11755	0,25045	0,0436	1,02874	1,20635
KM409663	3	0,26733	0,02055	0,01187	0,21628	0,31838	1,28567	0,00404	0,00233	1,27563	1,29571
KM409669	3	0,07067	0,00611	0,00353	0,05549	0,08585	0,98967	0,01589	0,00917	0,95021	1,02913
KM409673	3	0,23033	0,00551	0,00318	0,21665	0,24402	1,11733	0,03371	0,01946	1,03359	1,20107
KM409675	3	0,124	0,00819	0,00473	0,10367	0,14433	1,51067	0,01419	0,00819	1,47542	1,54592
KM409680	3	0,20633	0,00416	0,0024	0,19599	0,21668	0,62333	0,00569	0,00328	0,60921	0,63746
KM409682	3	0,176	0,001	0,00058	0,17352	0,17848	1,51433	0,052	0,03002	1,38515	1,64352
KM409685	3	0,079	0,00458	0,00265	0,06762	0,09038	1,17967	0,02859	0,01651	1,10865	1,25069
KM659864	3	0,03333	0,0138	0,00797	-0,0009	0,06761	1,05267	0,02603	0,01503	0,98802	1,11732
KM409696	3	0,04067	0,00289	0,00167	0,0335	0,04784	1,104	0,00985	0,00569	1,07953	1,12847
KM409697	3	0,09033	0,00551	0,00318	0,07665	0,10402	0,91867	0,05345	0,03086	0,78588	1,05145
KM409705	3	0,00867	0,00577	0,00333	-0,0057	0,02301	0,997	0,005	0,00289	0,98458	1,00942
Level	N	**30 °C**					**37 °C**				
Factor		Mean	Std.Dev.	Std.Err	-95,00%	95,00%	Mean	Std.Dev.	Std.Err	-95,00%	95,00%
	33	1,25215	0,21233	0,03696	1,17686	1,32744	1,40491	0,13339	0,02322	1,35761	1,45221
KM409663	3	1,275	0,01179	0,00681	1,24571	1,30429	1,567	0,01253	0,00723	1,53587	1,59813
KM409669	3	1,58633	0,02219	0,01281	1,53121	1,64145	1,53833	0,03302	0,01906	1,45631	1,62036
KM409673	3	1,51867	0,02754	0,0159	1,45026	1,58707	1,534	0,00656	0,00379	1,51771	1,55029
KM409675	3	1,58433	0,03717	0,02146	1,49201	1,67666	1,53667	0,0125	0,00722	1,50561	1,56773
KM409680	3	1,17033	0,01301	0,00751	1,13801	1,20266	1,33867	0,02421	0,01398	1,27852	1,39882
KM409682	3	1,28333	0,01102	0,00636	1,25597	1,3107	1,51267	0,0335	0,01934	1,42945	1,59589
KM409685	3	0,99433	0,04104	0,0237	0,89238	1,09628	1,21867	0,067	0,03868	1,05222	1,38511
KM659864	3	1,115	0,0344	0,01986	1,02956	1,20044	1,35533	0,00808	0,00467	1,33525	1,37541
KM409696	3	1,086	0,0311	0,01795	1,00875	1,16325	1,21167	0,03859	0,02228	1,1158	1,30753
KM409697	3	1,08233	0,06599	0,0381	0,91841	1,24626	1,31767	0,02286	0,0132	1,26089	1,37444
KM409705	3	1,078	0,01312	0,00757	1,04542	1,11058	1,32333	0,02578	0,01488	1,25931	1,38736
Level	N	**45 °C**									
Factor		Mean	Std.Dev.	Std.Err	-95,00%	95,00%					

	33	1,06746	0,34071	0,05931	0,94665	1,18826
KM409663	3	1,31	0,03534	0,0204	1,22221	1,39779
KM409669	3	1,351	0,08945	0,05164	1,1288	1,5732
KM409673	3	1,17267	0,03371	0,01946	1,08893	1,25641
KM409675	3	1,41267	0,03785	0,02185	1,31865	1,50668
KM409680	3	1,243	0,04924	0,02843	1,12067	1,36533
KM409682	3	1,10667	0,01626	0,00939	1,06628	1,14706
KM409685	3	0,11567	0,01274	0,00736	0,08402	0,14732
KM659864	3	0,944	0,016	0,00924	0,90425	0,98375
KM409696	3	1,03633	0,01955	0,01129	0,98776	1,08491
KM409697	3	1,03433	0,00751	0,00433	1,01569	1,05298
KM409705	3	1,01567	0,01914	0,01105	0,96812	1,06321

Table B. 4: Tukey HSD analysis for the South African *L. garvieae* isolates at 10 °C.

Cell No.	**Tukey HSD test; variable 10 °C** (Approximate Probabilities for Post Hoc Tests); Error: Between MS = ,00008, df = 22,000											
	isolates	KM409663	KM409669	KM409673	KM409675	KM409680	KM409682	KM409685	KM659864	KM409696	KM409697	KM409705
		0,26733	0,07067	0,23033	0,124	0,20633	0,176	0,079	0,03333	0,04067	0,09033	0,00867
1	KM409663	a	0,000201	0,001662	0,000201	0,000201	0,000201	0,000201	0,000201	0,000201	0,000201	0,000201
2	KM409669	0,000201	b	0,000201	0,000205	0,000201	0,000201	0,98139	0,001507	0,014141	0,250159	0,000201
3	KM409673	0,001662	0,000201	c	0,000201	0,083155	0,000204	0,000201	0,000201	0,000201	0,000201	0,000201
4	KM409675	0,000201	0,000205	0,000201	d	0,000201	0,000208	0,000294	0,000201	0,000201	0,004544	0,000201
5	KM409680	0,000201	0,000201	0,083155	0,000201	c	0,012761	0,000201	0,000201	0,000201	0,000201	0,000201
6	KM409682	0,000201	0,000201	0,000204	0,000208	0,012761	e	0,000201	0,000201	0,000201	0,000201	0,000201
7	KM409685	0,000201	0,98139	0,000201	0,000294	0,000201	0,000201	b	0,000275	0,001139	0,878209	0,000201
8	KM659864	0,000201	0,001507	0,000201	0,000201	0,000201	0,000201	0,000275	f	0,99264	0,000202	0,069003
9	KM409696	0,000201	0,014141	0,000201	0,000201	0,000201	0,000201	0,001139	0,99264	f	0,000218	0,007613
10	KM409697	0,000201	0,250159	0,000201	0,004544	0,000201	0,000201	0,878209	0,000202	0,000218	b	0,000201
11	KM409705	0,000201	0,000201	0,000201	0,000201	0,000201	0,000201	0,000201	0,069003	0,007613	0,000201	f

Table B. 5: Tukey HSD analysis for the South African *L. garvieae* isolates at 24 °C.

Cell No.	**Tukey HSD test; variable 24 °C** (Approximate Probabilities for Post Hoc Tests); Error:Between MS = ,00080, df = 22,000											
	isolates	KM409663	KM409669	KM409673	KM409675	KM409680	KM409682	KM409685	KM659864	KM409696	KM409697	KM409705
		1,2857	0,98967	1,1173	1,5107	0,62333	1,5143	1,1797	1,0527	1,104	0,91867	0,997
1	KM409663	g	0,000201	0,000207	0,000201	0,000201	0,000201	0,005448	0,000201	0,000202	0,000201	0,000201
2	KM409669	0,000201	h	0,000761	0,000201	0,000201	0,000201	0,000202	0,250515	0,002456	0,136242	1
3	KM409673	0,000207	0,000761	i	0,000201	0,000201	0,000201	0,262568	0,222148	0,999939	0,000201	0,001416
4	KM409675	0,000201	0,000201	0,000201	j	0,000201	1	0,000201	0,000201	0,000201	0,000201	0,000201
5	KM409680	0,000201	0,000201	0,000201	0,000201	k	0,000201	0,000201	0,000201	0,000201	0,000201	0,000201
6	KM409682	0,000201	0,000201	0,000201	1	0,000201	j	0,000201	0,000201	0,000201	0,000201	0,000201
7	KM409685	0,005448	0,000202	0,262568	0,000201	0,000201	0,000201	i	0,000802	0,092471	0,000201	0,000202
8	KM659864	0,000201	0,250515	0,222148	0,000201	0,000201	0,000201	0,000802	h i	0,514522	0,000486	0,404674
9	KM409696	0,000202	0,002456	0,999939	0,000201	0,000201	0,000201	0,092471	0,514522	i	0,000202	0,004946
10	KM409697	0,000201	0,136242	0,000201	0,000201	0,000201	0,000201	0,000201	0,000486	0,000202	h	0,073426
11	KM409705	0,000201	1	0,001416	0,000201	0,000201	0,000201	0,000202	0,404674	0,004946	0,073426	h

Table B. 6: Tukey HSD analysis for the South African *L. garvieae* isolates at 30 °C.

Cell No.	**Tukey HSD test; variable 30 °C** (Approximate Probabilities for Post Hoc Tests); Error: Between MS = ,00104, df = 22,000											
	isolates	KM409663	KM409669	KM409673	KM409675	KM409680	KM409682	KM409685	KM659864	KM409696	KM409697	KM409705
		1,275	1,5863	1,5187	1,5843	1,1703	1,2833	0,99433	1,115	1,086	1,0823	1,078
1	KM409663	l	0,000201	0,000201	0,000201	0,021075	1	0,000201	0,000341	0,000209	0,000206	0,000204
2	KM409669	0,000201	m	0,31942	1	0,000201	0,000201	0,000201	0,000201	0,000201	0,000201	0,000201
3	KM409673	0,000201	0,31942	m	0,35756	0,000201	0,000201	0,000201	0,000201	0,000201	0,000201	0,000201
4	KM409675	0,000201	1	0,35756	m	0,000201	0,000201	0,000201	0,000201	0,000201	0,000201	0,000201
5	KM409680	0,021075	0,000201	0,000201	0,000201	n	0,01044	0,00023	0,587102	0,105611	0,080264	0,057372
6	KM409682	1	0,000201	0,000201	0,000201	0,01044	l	0,000201	0,000263	0,000204	0,000203	0,000203
7	KM409685	0,000201	0,000201	0,000201	0,000201	0,00023	0,000201	o	0,00544	0,060448	0,080264	0,110906
8	KM659864	0,000341	0,000201	0,000201	0,000201	0,587102	0,000263	0,00544	n	0,986642	0,969664	0,934043
9	KM409696	0,000209	0,000201	0,000201	0,000201	0,105611	0,000204	0,060448	0,986642	n o	1	1
10	KM409697	0,000206	0,000201	0,000201	0,000201	0,080264	0,000203	0,080264	0,969664	1	n o	1
11	KM409705	0,000204	0,000201	0,000201	0,000201	0,057372	0,000203	0,110906	0,934043	1	1	n o

Table B. 7: Tukey HSD analysis for the South African *L. garvieae* isolates at 37 °C.

Cell No.	**Tukey HSD test; variable 37 °C** (Approximate Probabilities for Post Hoc Tests); Error: Between MS = ,00094, df = 22,000											
	isolates	KM409663	KM409669	KM409673	KM409675	KM409680	KM409682	KM409685	KM659864	KM409696	KM409697	KM409705
		1,567	1,5383	1,534	1,5367	1,3387	1,5127	1,2187	1,3553	1,2117	1,3177	1,3233
1	KM409663	p	0,982757	0,956054	0,974577	0,000201	0,548304	0,000201	0,000202	0,000201	0,000201	0,000201
2	KM409669	0,982757	p	1	1	0,000202	0,992281	0,000201	0,000207	0,000201	0,000201	0,000201
3	KM409673	0,956054	1	p	1	0,000203	0,998209	0,000201	0,00021	0,000201	0,000201	0,000202
4	KM409675	0,974577	1	1	p	0,000202	0,995383	0,000201	0,000208	0,000201	0,000201	0,000201
5	KM409680	0,000201	0,000202	0,000203	0,000202	q	0,000216	0,003511	0,999783	0,001913	0,998439	0,999897
6	KM409682	0,548304	0,992281	0,998209	0,995383	0,000216	p	0,000201	0,000287	0,000201	0,000203	0,000204
7	KM409685	0,000201	0,000201	0,000201	0,000201	0,003511	0,000201	r	0,000878	1	0,022608	0,013733
8	KM659864	0,000202	0,000207	0,00021	0,000208	0,999783	0,000287	0,000878	q	0,000541	0,904374	0,963851
9	KM409696	0,000201	0,000201	0,000201	0,000201	0,001913	0,000201	1	0,000541	r	0,012202	0,007363
10	KM409697	0,000201	0,000201	0,000201	0,000201	0,998439	0,000203	0,022608	0,904374	0,012202	q	1
11	KM409705	0,000201	0,000201	0,000202	0,000201	0,999897	0,000204	0,013733	0,963851	0,007363	1	q

Table B. 8: Tukey HSD analysis for the South African *L. garvieae* isolates at 45 °C.

Cell No.	**Tukey HSD test; variable 45 °C** (Approximate Probabilities for Post Hoc Tests); Error: Between MS = ,00143, df = 22,000											
	isolates	KM409663	KM409669	KM409673	KM409675	KM409680	KM409682	KM409685	KM659864	KM409696	KM409697	KM409705
		1,31	1,351	1,1727	1,4127	1,243	1,1067	0,11567	0,944	1,0363	1,0343	1,0157
1	KM409663	s	0,953382	0,007415	0,083476	0,545686	0,000239	0,000201	0,000201	0,000201	0,000201	0,000201
2	KM409669	0,953382	s	0,000502	0,652689	0,058764	0,000202	0,000201	0,000201	0,000201	0,000201	0,000201
3	KM409673	0,007415	0,000502	t	0,000203	0,480042	0,565723	0,000201	0,000205	0,007972	0,006897	0,001824
4	KM409675	0,083476	0,652689	0,000203	s	0,000801	0,000201	0,000201	0,000201	0,000201	0,000201	0,000201
5	KM409680	0,545686	0,058764	0,480042	0,000801	s t	0,007972	0,000201	0,000201	0,00023	0,000225	0,000206
6	KM409682	0,000239	0,000202	0,565723	0,000201	0,007972	t u	0,000201	0,001247	0,480042	0,441809	0,171055
7	KM409685	0,000201	0,000201	0,000201	0,000201	0,000201	0,000201	v	0,000201	0,000201	0,000201	0,000201
8	KM659864	0,000201	0,000201	0,000205	0,000201	0,000201	0,001247	0,000201	w	0,158214	0,177855	0,454369
9	KM409696	0,000201	0,000201	0,007972	0,000201	0,00023	0,480042	0,000201	0,158214	u w	1	0,999769
10	KM409697	0,000201	0,000201	0,006897	0,000201	0,000225	0,441809	0,000201	0,177855	1	u w	0,999907
11	KM409705	0,000201	0,000201	0,001824	0,000201	0,000206	0,171055	0,000201	0,454369	0,999769	0,999907	u w

Table B. 9: Descriptive statistics of the *L. garvieae* isolates from Lesotho at different temperatures.

Descriptive Statistics_ Temperature (*L. garvieae*_ Lesotho isolates)											
Level	N	**10 °C**					**24 °C**				
Factor		Mean	Std.Dev.	Std.Err	-95,00%	95,00%	Mean	Std.Dev.	Std.Err	-95,00%	95,00%
Total	30	0,14883	0,22877	0,04177	0,06341	0,23426	1,13463	0,16212	0,0296	1,0741	1,19517
KM409665	3	0,812	0,00656	0,00379	0,79571	0,82829	1,238	0,02095	0,0121	1,18595	1,29005
KM409664	3	0,15267	0,01069	0,00617	0,12611	0,17923	1,07667	0,03661	0,02114	0,98572	1,16761
KM409668	3	0,144	0,02166	0,0125	0,0902	0,1978	1,2	0,00794	0,00458	1,18028	1,21972
KM409689	3	0,058	0,01136	0,00656	0,02979	0,08621	1,051	0,01778	0,01026	1,00684	1,09516
KM409691	3	0,071	0,01312	0,00757	0,03842	0,10358	0,997	0,004	0,00231	0,98706	1,00694
KM409694	3	0,08367	0,00757	0,00437	0,06486	0,10248	1,209	0,01735	0,01002	1,1659	1,2521
KM409695	3	0,037	0,00917	0,00529	0,01423	0,05977	1,01567	0,00306	0,00176	1,00808	1,02326
KM409701	3	0,03	0,007	0,00404	0,01261	0,04739	1,531	0,04251	0,02454	1,4254	1,6366
KM409702	3	0,03767	0,0205	0,01184	-0,0133	0,0886	0,99133	0,00451	0,0026	0,98013	1,00254
KM409703	3	0,06233	0,00764	0,00441	0,04336	0,08131	1,03667	0,00058	0,00033	1,03523	1,0381
Level of	N	**30 °C**					**37 °C**				
Factor		Mean	Std.Dev.	Std.Err	-95,00%	95,00%	Mean	Std.Dev.	Std.Err	-95,00%	95,00%
	30	1,3614	0,15024	0,02743	1,3053	1,4175	1,53357	0,06473	0,01182	1,5094	1,55774
KM409665	3	1,57333	0,00493	0,00285	1,56108	1,58559	1,362	0,03799	0,02193	1,26764	1,45637
KM409664	3	1,63767	0,04688	0,02706	1,52122	1,75411	1,56667	0,02376	0,01372	1,50765	1,62568
KM409668	3	1,50133	0,00907	0,00524	1,47879	1,52387	1,576	0,00889	0,00513	1,55392	1,59808
KM409689	3	1,304	0,04071	0,0235	1,20288	1,40512	1,595	0,01411	0,00815	1,55996	1,63004
KM409691	3	1,25067	0,05168	0,02984	1,1223	1,37904	1,53633	0,00058	0,00033	1,5349	1,53777
KM409694	3	1,297	0,02352	0,01358	1,23858	1,35542	1,513	0,00781	0,00451	1,4936	1,5324
KM409695	3	1,175	0,03831	0,02212	1,07982	1,27018	1,562	0,01179	0,00681	1,53271	1,59129
KM409701	3	1,30033	0,0205	0,01184	1,2494	1,35126	1,55733	0,01856	0,01071	1,51124	1,60343
KM409702	3	1,319	0,00361	0,00208	1,31004	1,32796	1,546	0,01229	0,0071	1,51547	1,57653
KM409703	3	1,25567	0,01504	0,00869	1,21829	1,29304	1,52133	0,02122	0,01225	1,46862	1,57405
Level of	N	**45 °C**									
Factor		Mean	Std.Dev.	Std.Err	-95,00%	95,00%					
	30	1,15823	0,27578	0,05035	1,05526	1,26121					
KM409665	3	1,504	0,03158	0,01823	1,42556	1,58244					
KM409664	3	1,21633	0,01206	0,00696	1,18639	1,24628					
KM409668	3	0,749	0,00872	0,00503	0,72734	0,77066					
KM409689	3	1,43467	0,02969	0,01714	1,36092	1,50841					
KM409691	3	1,34033	0,05133	0,02963	1,21283	1,46783					
KM409694	3	0,765	0,05407	0,03121	0,6307	0,8993					
KM409695	3	1,167	0,037	0,02136	1,07509	1,25891					

KM409701	3	0,824	0,06846	0,03953	0,65393	0,99407
KM409702	3	1,386	0,021	0,01212	1,33383	1,43817
KM409703	3	1,196	0,09539	0,05508	0,95903	1,43297

Table B. 10: Tukey HSD analysis for the *L. garvieae* isolates from Lesotho at 10 °C.

Cell No.	**Tukey HSD test; variable 10 °C** (Approximate Probabilities for Post Hoc Tests); Error: Between MS = ,00016, df = 20,000										
	Isolates	KM409665	KM409664	KM409668	KM409689	KM409691	KM409694	KM409695	KM409701	KM409702	KM409703
		0.812	0.15267	0.144	0.058	0.071	0.08367	0.037	0.03	0.03767	0.06233
1	KM409665	a	0.000179	0.000179	0.000179	0.000179	0.000179	0.000179	0.000179	0.000179	0.000179
2	KM409664	0.000179	b	0.996792	0.000179	0.000187	0.000225	0.000179	0.000179	0.000179	0.000179
3	KM409668	0.000179	0.996792	b	0.000186	0.0002	0.000484	0.000179	0.000179	0.000179	0.000187
4	KM409689	0.000179	0.000179	0.000186	c	0.951773	0.330786	0.586653	0.232613	0.626231	0.999988
5	KM409691	0.000179	0.000187	0.0002	0.951773	c	0.958602	0.081499	0.020369	0.092312	0.996792
6	KM409694	0.000179	0.000225	0.000484	0.330786	0.958602	c d	0.006286	0.001519	0.007224	0.566865
7	KM409695	0.000179	0.000179	0.000179	0.586653	0.081499	0.006286	c	0.999382	1	0.346709
8	KM409701	0.000179	0.000179	0.000179	0.232613	0.020369	0.001519	0.999382	c	0.998744	0.110913
9	KM409702	0.000179	0.000179	0.000179	0.626231	0.092312	0.007224	1	0.998744	c	0.379904
10	KM409703	0.000179	0.000179	0.000187	0.999988	0.996792	0.566865	0.346709	0.110913	0.379904	c d

Table B. 11: Tukey HSD analysis for the *L. garvieae* isolates from Lesotho at 24 °C.

Cell No.	**Tukey HSD test; variable 24 °C** (Approximate Probabilities for Post Hoc Tests) Error: Between MS = ,00043, df = 20,000										
	Isolates	KM409665	KM409664	KM409668	KM409689	KM409691	KM409694	KM409695	KM409701	KM409702	KM409703
		1.238	1.0767	1.2	1.051	0.997	1.209	1.0157	1.531	0.99133	1.0367
1	KM409665	e	0.000179	0.463099	0.000179	0.000179	0.777905	0.000179	0.000179	0.000179	0.000179
2	KM409664	0.000179	f	0.000194	0.871489	0.004326	0.000188	0.044628	0.000179	0.002145	0.396957
3	KM409668	0.463099	0.000194	e	0.000179	0.000179	0.999916	0.000179	0.000179	0.000179	0.000179
4	KM409689	0.000179	0.871489	0.000179	f g	0.100837	0.000179	0.557142	0.000179	0.052349	0.996642
5	KM409691	0.000179	0.004326	0.000179	0.100837	g	0.000179	0.978951	0.000179	0.999998	0.407639
6	KM409694	0.777905	0.000188	0.999916	0.000179	0.000179	e	0.000179	0.000179	0.000179	0.000179
7	KM409695	0.000179	0.044628	0.000179	0.557142	0.978951	0.000179	g	0.000179	0.901543	0.956424
8	KM409701	0.000179	0.000179	0.000179	0.000179	0.000179	0.000179	0.000179	h	0.000179	0.000179
9	KM409702	0.000179	0.002145	0.000179	0.052349	0.999998	0.000179	0.901543	0.000179	g	0.248424
10	KM409703	0.000179	0.396957	0.000179	0.996642	0.407639	0.000179	0.956424	0.000179	0.248424	f g

Table B. 12: Tukey HSD analysis for the *L. garvieae* isolates from Lesotho at 30 °C.

Cell No.	**Tukey HSD test; variable 30 °C** (Approximate Probabilities for Post Hoc Tests); Error: Between MS = ,00093, df = 20,000										
	Isolates	KM409665	KM409664	KM409668	KM409689	KM409691	KM409694	KM409695	KM409701	KM409702	KM409703
		1.5733	1.6377	1.5013	1.304	1.2507	1.297	1.175	1.3003	1.319	1.2557
1	KM409665	i	0.287028	0.172834	0.000179	0.000179	0.000179	0.000179	0.000179	0.000179	0.000179
2	KM409664	0.287028	i	0.000905	0.000179	0.000179	0.000179	0.000179	0.000179	0.000179	0.000179
3	KM409668	0.172834	0.000905	i	0.000187	0.000179	0.000186	0.000179	0.000187	0.000193	0.000179
4	KM409689	0.000179	0.000179	0.000187	j	0.522778	1	0.001595	1	0.999763	0.645223
5	KM409691	0.000179	0.000179	0.000179	0.522778	j	0.69344	0.132803	0.61256	0.222002	1
6	KM409694	0.000179	0.000179	0.000186	1	0.69344	j	0.002844	1	0.995373	0.804676
7	KM409695	0.000179	0.000179	0.000179	0.001595	0.132803	0.002844	k	0.002156	0.000536	0.091192
8	KM409701	0.000179	0.000179	0.000187	1	0.61256	1	0.002156	j	0.998664	0.732365
9	KM409702	0.000179	0.000179	0.000193	0.999763	0.222002	0.995373	0.000536	0.998664	j	0.30529
10	KM409703	0.000179	0.000179	0.000179	0.645223	1	0.804676	0.091192	0.732365	0.30529	j k

Table B. 13: Tukey HSD analysis for the *L. garvieae* isolates from Lesotho at 30 °C.

Cell No.	**Tukey HSD test; variable 37 °C** (Approximate Probabilities for Post Hoc Tests) Error: Between MS = ,00034, df = 20,000										
	Isolates	KM409665	KM409664	KM409668	KM409689	KM409691	KM409694	KM409695	KM409701	KM409702	KM409703
		1.362	1.5667	1.576	1.595	1.5363	1.513	1.562	1.5573	1.546	1.5213
1	KM409665	l	0.000179	0.000179	0.000179	0.000179	0.000179	0.000179	0.000179	0.000179	0.000179
2	KM409664	0.000179	m	0.99971	0.685291	0.605098	0.049337	0.999999	0.99971	0.923989	0.142698
3	KM409668	0.000179	0.99971	m	0.952644	0.269428	0.01356	0.993502	0.95732	0.618595	0.043128
4	KM409689	0.000179	0.685291	0.952644	m n	0.024916	0.000992	0.49805	0.329624	0.090812	0.003002
5	KM409691	0.000179	0.605098	0.269428	0.024916	m	0.859285	0.784992	0.917179	0.999616	0.989482
6	KM409694	0.000179	0.049337	0.01356	0.000992	0.859285	o	0.090812	0.16061	0.49805	0.999885
7	KM409695	0.000179	0.999999	0.993502	0.49805	0.784992	0.090812	m n o	0.999999	0.983767	0.242445
8	KM409701	0.000179	0.99971	0.95732	0.329624	0.917179	0.16061	0.999999	m n o	0.998662	0.385655
9	KM409702	0.000179	0.923989	0.618595	0.090812	0.999616	0.49805	0.983767	0.998662	m n o	0.81863
10	KM409703	0.000179	0.142698	0.043128	0.003002	0.989482	0.999885	0.242445	0.385655	0.81863	m o

Table B. 14: Tukey HSD analysis for the *L. garvieae* isolates from Lesotho at 45 °C.

Cell No.	**Tukey HSD test; variable 45 °C** (Approximate Probabilities for Post Hoc Tests); Error: Between MS = ,00233, df = 20,000										
	temp	KM409665	KM409664	KM409668	KM409689	KM409691	KM409694	KM409695	KM409701	KM409702	KM409703
		1.504	1.2163	0.749	1.4347	1.3403	0.765	1.167	0.824	1.386	1.196
1	KM409665	p	0.000194	0.000179	0.750293	0.013812	0.000179	0.000179	0.000179	0.142793	0.000188
2	KM409664	0.000194	q	0.000179	0.00079	0.10782	0.000179	0.953358	0.000179	0.009968	0.999933
3	KM409668	0.000179	0.000179	r	0.000179	0.000179	0.999991	0.000179	0.666415	0.000179	0.000179
4	KM409689	0.750293	0.00079	0.000179	p s	0.377386	0.000179	0.000215	0.000179	0.956942	0.000365
5	KM409691	0.013812	0.10782	0.000179	0.377386	q s	0.000179	0.008159	0.000179	0.970718	0.038818
6	KM409694	0.000179	0.000179	0.999991	0.000179	0.000179	r	0.000179	0.877692	0.000179	0.000179
7	KM409695	0.000179	0.953358	0.000179	0.000215	0.008159	0.000179	q	0.000179	0.000767	0.998828
8	KM409701	0.000179	0.000179	0.666415	0.000179	0.000179	0.877692	0.000179	r	0.000179	0.000179
9	KM409702	0.142793	0.009968	0.000179	0.956942	0.970718	0.000179	0.000767	0.000179	p s	0.003309
10	KM409703	0.000188	0.999933	0.000179	0.000365	0.038818	0.000179	0.998828	0.000179	0.003309	q

Table B. 15: Descriptive statistics of the *Weissella* species at different temperatures.

Descriptive Statistics_ Temperature (*Weissella* species)											
Level	N	**10 °C**					**24 °C**				
Factor		Mean	Std.Dev.	Std.Err	-95,00%	95,00%	Mean	Std.Dev.	Std.Err	-95,00%	95,00%
Total	6	0,090667	0,033703	0,013759	0,055298	0,126035	0,256167	0,058198	0,023759	0,195092	0,317241
KM409656	3	0,084	0,051971	0,030006	-0,045103	0,213103	0,308	0,018682	0,010786	0,261592	0,354408
KM409657	3	0,097333	0,002309	0,001333	0,091596	0,10307	0,204333	0,007638	0,00441	0,18536	0,223306
Level of	N	**30 °C**					**37 °C**				
Factor		Mean	Std.Dev.	Std.Err	-95,00%	95,00%	Mean	Std.Dev.	Std.Err	-95,00%	95,00%
Total	6	0,211167	0,052347	0,02137	0,156232	0,266101	0,392883	0,073772	0,030117	0,315465	0,470302
KM409656	3	0,257	0,004359	0,002517	0,246172	0,267828	0,335	0,014422	0,008327	0,299173	0,370827
KM409657	3	0,165333	0,023007	0,013283	0,10818	0,222486	0,450767	0,057847	0,033398	0,307067	0,594466
Level	N	**45 °C**									
Factor		Mean	Std.Dev.	Std.Err	-95,00%	95,00%					
Total	6	0,087	0,024722	0,010093	0,061055	0,112945					
KM409656	3	0,100667	0,023629	0,013642	0,041969	0,159365					
KM409657	3	0,073333	0,020232	0,011681	0,023074	0,123592					

Table B. 16: Tukey HSD analysis for *Weissella* species at different temperatures.

Cell No.	Tukey HSD test; variable 10 °C Approximate Probabilities Error: Between MS = ,00135, df = 4,0000		
	isolates	KM409656	KM409657
		0,084	0,09733
1	KM409656	a	0,680246
2	KM409657	0,680246	a

Cell No.	Tukey HSD test; variable 24 °C Approximate Probabilities Error: Between MS = ,00020, df = 4,0000		
	isolates	KM409656	KM409657
		0,308	0,20433
1	KM409656	b	0,001093
2	KM409657	0,001093	c

Cell No.	Tukey HSD test; variable 30 °C Approximate Probabilities Error: Between MS = ,00027, df = 4,0000		
	isolates	KM409656	KM409657
		0,257	0,16533
1	KM409656	d	0,002675
2	KM409657	0,002675	e

Cell No.	Tukey HSD test; variable 37 °C Approximate Probabilities Error: Between MS = ,00178, df = 4,0000		
	isolates	KM409656	KM409657
		0,335	0,45077
1	KM409656	f	0,028402
2	KM409657	0,028402	g

Cell No.	Tukey HSD test; variable 45 °C Approximate Probabilities Error: Between MS = ,00048, df = 4,0000		
	isolates	KM409656	KM409657
		0,10067	0,07333
1	KM409656	h	0,202898
2	KM409657	0,202898	h

Table B. 17: Descriptive statistics of *C. maltaromaticum* sp. at different temperatures.

Descriptive Statistics_ Temperature (*C. maltaromaticum*)											
Level	N	**10 °C**					**24 °C**				
Factor		Mean	Std.Dev.	Std.Err	-95,00%	95,00%	Mean	Std.Dev.	Std.Err	-95,00%	95,00%
Total	9	0,383333	0,050887	0,016962	0,344218	0,422449	1,093889	0,059202	0,019734	1,048382	1,139395
KM409658	3	0,423667	0,005859	0,003383	0,409111	0,438222	1,167	0,02512	0,014503	1,104599	1,229401
KM409660	3	0,328	0,052849	0,030512	0,196716	0,459284	1,044667	0,028937	0,016707	0,972784	1,116549
KM409659	3	0,398333	0,012662	0,007311	0,366878	0,429788	1,07	0,006557	0,003786	1,05371	1,08629
Level of	N	**30 °C**					**37 °C**				
Factor		Mean	Std.Dev.	Std.Err	-95,00%	95,00%	Mean	Std.Dev.	Std.Err	-95,00%	95,00%
Total	9	1,108444	0,153459	0,051153	0,990485	1,226404	0,977556	0,095681	0,031894	0,904009	1,051102
KM409658	3	0,993667	0,016563	0,009563	0,952522	1,034811	0,873333	0,01914	0,01105	0,825787	0,920879
KM409660	3	1,025333	0,031086	0,017947	0,948112	1,102555	0,971	0,03629	0,020952	0,880849	1,061151
KM409659	3	1,306333	0,064003	0,036952	1,147342	1,465325	1,088333	0,013051	0,007535	1,055912	1,120754
Level of	N	**45 °C**									
Factor		Mean	Std.Dev.	Std.Err	-95,00%	95,00%					
Total	9	1,341444	0,087354	0,029118	1,274298	1,408591					
KM409658	3	1,279667	0,019757	0,011407	1,230588	1,328745					
KM409660	3	1,290667	0,015308	0,008838	1,25264	1,328694					
KM409659	3	1,454	0,036097	0,020841	1,36433	1,54367					

Table B. 18: Tukey HSD analysis for *C. maltaromaticum* sp. at different temperatures.

Cell No.	Tukey HSD test; variable 24 °C Approximate Probabilities for Post Hoc Tests Error: Between MS = ,00050, df = 6,0000			
	temp	KM409658	KM409660	KM409659
		1,167	1,0447	1,07
1	KM409658	c	0,001517	0,004581
2	KM409660	0,001517	d	0,406723
3	KM409659	0,004581	0,406723	d

Cell No.	Tukey HSD test; variable 30 °C Approximate Probabilities for Post Hoc Tests Error: Between MS = ,00178, df = 6,0000			
	temp	KM409658	KM409660	KM409659
		0,99367	1,0253	1,3063
1	KM409658	e	0,648981	0,00044
2	KM409660	0,648981	e	0,000632
3	KM409659	0,00044	0,000632	f

Cell No.	Tukey HSD test; variable 37 °C Approximate Probabilities for Post Hoc Tests Error: Between MS = ,00062, df = 6,0000			
	temp	KM409658	KM409660	KM409659
		0,87333	0,971	1,0883
1	KM409658	g	0,007227	0,000299
2	KM409660	0,007227	h	0,002992
3	KM409659	0,000299	0,002992	i

Cell No.	Tukey HSD test; variable 45 °C Approximate Probabilities for Post Hoc Tests Error: Between MS = ,00064, df = 6,0000			
	temp	KM409658	KM409660	KM409659
		1,2797	1,2907	1,454
1	KM409658	j	0,85932	0,000558
2	KM409660	0,85932	j	0,000717
3	KM409659	0,000558	0,000717	k

Cell No.	Tukey HSD test; variable 10 °C Approximate Probabilities for Post Hoc Tests Error: Between MS = ,00100, df = 6,0000			
	temp	KM409658	KM409660	KM409659
		0,42367	0,328	0,39833
1	KM409658	a	0,023292	0,61301
2	KM409660	0,023292	b	0,076413
3	KM409659	0,61301	0,076413	a b

Statistical Analysis Results_pH

Table B. 19: Descriptive statistics of the South African *L. garvieae* isolates at different pH.

Descriptive Statistics_ pH (*L. garvieae*_ South African isolates)											
Level	N	**pH 4.5**					**pH 5**				
Factor		Mean	Std.Dev.	Std.Err	-95,00%	95,00%	Mean	Std.Dev.	Std.Err	-95,00%	95,00%
Total	33	0,145909	0,20162	0,035097	0,074418	0,2174	0,586212	0,212453	0,036983	0,51088	0,661545
KM409663	3	0,241	0,00781	0,004509	0,221598	0,260402	0,455667	0,014844	0,00857	0,418793	0,49254
KM409669	3	0,346333	0,005859	0,003383	0,331778	0,360889	0,520333	0,010017	0,005783	0,495451	0,545216
KM409673	3	-0,114333	0,019858	0,011465	-0,163663	-0,065004	0,486333	0,002517	0,001453	0,480082	0,492585
KM409675	3	0,335333	0,007234	0,004177	0,317363	0,353304	1,229667	0,032517	0,018774	1,148891	1,310443
KM409680	3	-0,141	0,037323	0,021548	-0,233715	-0,048285	0,478667	0,010599	0,006119	0,452338	0,504995
KM409682	3	-0,099667	0,010599	0,006119	-0,125995	-0,073338	0,463	0,015	0,00866	0,425738	0,500262
KM409685	3	-0,089	0,001732	0,001	-0,093303	-0,084697	0,532667	0,040452	0,023355	0,432179	0,633154
KM659864	3	0,207333	0,01861	0,010745	0,161103	0,253563	0,600667	0,008963	0,005175	0,578402	0,622932
KM409696	3	0,296	0,008544	0,004933	0,274776	0,317224	0,528667	0,008737	0,005044	0,506963	0,55037
KM409697	3	0,312667	0,014048	0,00811	0,277771	0,347563	0,587667	0,012503	0,007219	0,556607	0,618727
KM409705	3	0,310333	0,009292	0,005364	0,287252	0,333415	0,565	0,006245	0,003606	0,549487	0,580513
Level	N	**pH 7**					**pH 8**				
Factor		Mean	Std.Dev.	Std.Err	-95,00%	95,00%	Mean	Std.Dev.	Std.Err	-95,00%	95,00%
Total	33	1,353273	0,157317	0,027385	1,29749	1,409055	1,457121	0,087678	0,015263	1,426032	1,48821
KM409663	3	1,217667	0,03402	0,019641	1,133157	1,302176	1,576	0,013856	0,008	1,541579	1,610421
KM409669	3	1,501667	0,017388	0,010039	1,458473	1,54486	1,483667	0,006429	0,003712	1,467696	1,499637
KM409673	3	1,302667	0,022234	0,012837	1,247435	1,357898	1,509667	0,008083	0,004667	1,489588	1,529746
KM409675	3	1,407	0,03005	0,017349	1,332352	1,481648	1,583667	0,018824	0,010868	1,536906	1,630427
KM409680	3	1,476	0,003606	0,002082	1,467043	1,484957	1,371667	0,01963	0,011333	1,322903	1,42043
KM409682	3	1,282	0,033151	0,01914	1,199648	1,364352	1,485	0,01044	0,006028	1,459065	1,510935

KM409685	3	0,959667	0,055582	0,03209	0,821594	1,097739	1,304333	0,005033	0,002906	1,29183	1,316837
KM659864	3	1,487	0,018248	0,010536	1,441669	1,532331	1,446333	0,046069	0,026598	1,331892	1,560775
KM409696	3	1,479	0,001	0,000577	1,476516	1,481484	1,505667	0,01701	0,009821	1,463412	1,547921
KM409697	3	1,418667	0,014978	0,008647	1,38146	1,455873	1,397	0,003464	0,002	1,388395	1,405605
KM409705	3	1,354667	0,004726	0,002728	1,342927	1,366406	1,365333	0,008963	0,005175	1,343068	1,387598

Level	N	**pH 9.5**				
Factor		Mean	Std.Dev.	Std.Err	-95,00%	95,00%
Total	33	1,186273	0,140977	0,024541	1,136285	1,236261
KM409663	3	1,338333	0,021548	0,012441	1,284804	1,391863
KM409669	3	1,315333	0,014224	0,008212	1,279998	1,350669
KM409673	3	1,385	0,005292	0,003055	1,371855	1,398145
KM409675	3	1,274333	0,044501	0,025693	1,163787	1,38488
KM409680	3	1,017333	0,019553	0,011289	0,96876	1,065907
KM409682	3	1,277667	0,029872	0,017247	1,203461	1,351873
KM409685	3	1,086333	0,016921	0,00977	1,044298	1,128368
KM659864	3	1,088333	0,0306	0,017667	1,01232	1,164347
KM409696	3	1,027333	0,032254	0,018622	0,947209	1,107457
KM409697	3	1,224	0,036497	0,021071	1,133337	1,314663
KM409705	3	1,015	0,105674	0,061011	0,752491	1,277509

Table B. 20: Tukey HSD analysis for *L. garvieae* isolates from South Africa at different pH 4.5.

Cell No.	**Tukey HSD test; variable pH 4.5** (Approximate Probabilities for Post Hoc Tests); Error: Between MS = ,00025, df = 22,000											
	isolates	KM409663	KM409669	KM409673	KM409675	KM409680	KM409682	KM409685	KM659864	KM409696	KM409697	KM409705
		0.241	0.34633	-0.1143	0.33533	-0.141	-0.0997	-0.089	0.20733	0.296	0.31267	0.31033
1	KM409663	a	0.000202	0.000201	0.000207	0.000201	0.000201	0.000201	0.302902	0.01134	0.000728	0.001027
2	KM409669	0.000202	b	0.000201	0.998187	0.000201	0.000201	0.000201	0.000201	0.025185	0.302902	0.226155
3	KM409673	0.000201	0.000201	c	0.000201	0.611162	0.983513	0.674682	0.000201	0.000201	0.000201	0.000201
4	KM409675	0.000207	0.998187	0.000201	b d	0.000201	0.000201	0.000201	0.000201	0.143128	0.792659	0.690264
5	KM409680	0.000201	0.000201	0.611162	0.000201	c	0.10675	0.018991	0.000201	0.000201	0.000201	0.000201
6	KM409682	0.000201	0.000201	0.983513	0.000201	0.10675	c	0.998604	0.000201	0.000201	0.000201	0.000201
7	KM409685	0.000201	0.000201	0.674682	0.000201	0.018991	0.998604	c	0.000201	0.000201	0.000201	0.000201
8	KM659864	0.302902	0.000201	0.000201	0.000201	0.000201	0.000201	0.000201	a	0.000219	0.000202	0.000202
9	KM409696	0.01134	0.025185	0.000201	0.143128	0.000201	0.000201	0.000201	0.000219	d	0.96109	0.98599
10	KM409697	0.000728	0.302902	0.000201	0.792659	0.000201	0.000201	0.000201	0.000202	0.96109	b d	1
11	KM409705	0.001027	0.226155	0.000201	0.690264	0.000201	0.000201	0.000201	0.000202	0.98599	1	b d

Table B. 21: Tukey HSD analysis for *L. garvieae* isolates from South Africa at different pH 5.

Cell No.	**Tukey HSD test; variable pH 5** (Approximate Probabilities for Post Hoc Tests); Error: Between MS = ,00034, df = 22,000											
	isolates	KM409663	KM409669	KM409673	KM409675	KM409680	KM409682	KM409685	KM659864	KM409696	KM409697	KM409705
		0.45567	0.52033	0.48633	1.2297	0.47867	0.463	0.53267	0.60067	0.52867	0.58767	0.565
1	KM409663	e	0.010032	0.623079	0.000201	0.892629	0.999987	0.001641	0.000201	0.002908	0.000201	0.000207
2	KM409669	0.010032	f	0.486255	0.000201	0.229279	0.029502	0.998649	0.001046	0.999957	0.00674	0.161565
3	KM409673	0.623079	0.486255	e f	0.000201	0.99998	0.884137	0.131605	0.000203	0.212633	0.000225	0.001304
4	KM409675	0.000201	0.000201	0.000201	g	0.000201	0.000201	0.000201	0.000201	0.000201	0.000201	0.000201
5	KM409680	0.892629	0.229279	0.99998	0.000201	e f	0.99096	0.047376	0.000202	0.081911	0.000207	0.000515
6	KM409682	0.999987	0.029502	0.884137	0.000201	0.99096	e	0.00476	0.000201	0.008641	0.000202	0.000222
7	KM409685	0.001641	0.998649	0.131605	0.000201	0.047376	0.00476	f	0.006103	1	0.041161	0.554083
8	KM659864	0.000201	0.001046	0.000203	0.000201	0.000202	0.000201	0.006103	h	0.00337	0.997902	0.421225
9	KM409696	0.002908	0.999957	0.212633	0.000201	0.081911	0.008641	1	0.00337	f	0.023173	0.396435
10	KM409697	0.000201	0.00674	0.000225	0.000201	0.000207	0.000202	0.041161	0.997902	0.023173	h	0.900745
11	KM409705	0.000207	0.161565	0.001304	0.000201	0.000515	0.000222	0.554083	0.421225	0.396435	0.900745	f h

Table B. 22: Tukey HSD analysis for *L. garvieae* isolates from South Africa at different pH 7.

Cell No.	**Tukey HSD test; variable pH 7** (Spreadsheet3 in Workbook2) (Approximate Probabilities for Post Hoc Tests); Error: Between MS = ,00069, df = 22,000											
	isolates	KM409663	KM409669	KM409673	KM409675	KM409680	KM409682	KM409685	KM659864	KM409696	KM409697	KM409705
		1.2177	1.5017	1.3027	1.407	1.476	1.282	0.95967	1.487	1.479	1.4187	1.3547
1	KM409663	i	0.000201	0.022389	0.000201	0.000201	0.158329	0.000201	0.000201	0.000201	0.000201	0.000268
2	KM409669	0.000201	j	0.000201	0.008255	0.976785	0.000201	0.000201	0.999728	0.990392	0.027413	0.000221
3	KM409673	0.022389	0.000201	k	0.003034	0.000202	0.995235	0.000201	0.000201	0.000202	0.000981	0.39991
4	KM409675	0.000201	0.008255	0.003034	l	0.105296	0.000477	0.000201	0.037001	0.080005	0.999965	0.391412
5	KM409680	0.000201	0.976785	0.000202	0.105296	j l	0.000201	0.000201	0.99998	1	0.276376	0.000622
6	KM409682	0.158329	0.000201	0.995235	0.000477	0.000201	i k	0.000201	0.000201	0.000201	0.000271	0.075141
7	KM409685	0.000201	0.000201	0.000201	0.000201	0.000201	0.000201	m	0.000201	0.000201	0.000201	0.000201
8	KM659864	0.000201	0.999728	0.000201	0.037001	0.99998	0.000201	0.000201	j n	0.999999	0.111785	0.000318
9	KM409696	0.000201	0.990392	0.000202	0.080005	1	0.000201	0.000201	0.999999	j l	0.219688	0.000499
10	KM409697	0.000201	0.027413	0.000981	0.999965	0.276376	0.000271	0.000201	0.111785	0.219688	l n	0.162844
11	KM409705	0.000268	0.000221	0.39991	0.391412	0.000622	0.075141	0.000201	0.000318	0.000499	0.162844	k l

Table B. 23: Tukey HSD analysis for *L. garvieae* isolates from South Africa at different pH 8.

Cell No.	**Tukey HSD test; variable pH 8** (Spreadsheet3 in Workbook2) (Approximate Probabilities for Post Hoc Tests); Error: Between MS = ,00033, df = 22,000											
	isolates	KM409663	KM409669	KM409673	KM409675	KM409680	KM409682	KM409685	KM659864	KM409696	KM409697	KM409705
		1.576	1.4837	1.5097	1.5837	1.3717	1.485	1.3043	1.4463	1.5057	1.397	1.3653
1	KM409663	o	0.000308	0.007481	0.999979	0.000201	0.000335	0.000201	0.000201	0.004114	0.000201	0.000201
2	KM409669	0.000308	p	0.799391	0.000229	0.000204	1	0.000201	0.354873	0.913692	0.00048	0.000202
3	KM409673	0.007481	0.799391	p	0.002397	0.000201	0.843097	0.000201	0.011724	1	0.000204	0.000201
4	KM409675	0.999979	0.000229	0.002397	o	0.000201	0.000237	0.000201	0.000201	0.00136	0.000201	0.000201
5	KM409680	0.000201	0.000204	0.000201	0.000201	q	0.000204	0.006441	0.002178	0.000201	0.821877	0.999997
6	KM409682	0.000335	1	0.843097	0.000237	0.000204	p	0.000201	0.310539	0.939874	0.000424	0.000202
7	KM409685	0.000201	0.000201	0.000201	0.000201	0.006441	0.000201	r	0.000201	0.000201	0.000302	0.016598
8	KM659864	0.000201	0.354873	0.011724	0.000201	0.002178	0.310539	0.000201	p s	0.021227	0.086877	0.000914
9	KM409696	0.004114	0.913692	1	0.00136	0.000201	0.939874	0.000201	0.021227	p	0.000207	0.000201
10	KM409697	0.000201	0.00048	0.000204	0.000201	0.821877	0.000424	0.000302	0.086877	0.000207	q s	0.575724
11	KM409705	0.000201	0.000202	0.000201	0.000201	0.999997	0.000202	0.016598	0.000914	0.000201	0.575724	q

Table B. 24: Tukey HSD analysis for *L. garvieae* isolates from South Africa at different pH 9.5.

Cell No.	**Tukey HSD test; variable pH 9.5** (Spreadsheet3 in Workbook2) (Approximate Probabilities for Post Hoc Tests); Error: Between MS = ,00170, df = 22,000											
	isolates	KM409663	KM409669	KM409673	KM409675	KM409680	KM409682	KM409685	KM659864	KM409696	KM409697	KM409705
		1.3383	1.3153	1.385	1.2743	1.0173	1.2777	1.0863	1.0883	1.0273	1.224	1.015
1	KM409663	t	0.999723	0.939537	0.711007	0.000201	0.767518	0.000205	0.000205	0.000201	0.072469	0.000201
2	KM409669	0.999723	t	0.608416	0.97336	0.000201	0.985167	0.000223	0.000226	0.000201	0.255976	0.000201
3	KM409673	0.939537	0.608416	t	0.090138	0.000201	0.109347	0.000201	0.000201	0.000201	0.003527	0.000201
4	KM409675	0.711007	0.97336	0.090138	t	0.000203	1	0.000685	0.000761	0.000206	0.906681	0.000203
5	KM409680	0.000201	0.000201	0.000201	0.000203	u	0.000203	0.620703	0.58381	1	0.000322	1
6	KM409682	0.767518	0.985167	0.109347	1	0.000203	t	0.00058	0.00064	0.000205	0.868942	0.000203
7	KM409685	0.000205	0.000223	0.000201	0.000685	0.620703	0.00058	u	1	0.794059	0.016539	0.577661
8	KM659864	0.000205	0.000226	0.000201	0.000761	0.58381	0.00064	1	u	0.76206	0.018859	0.540893
9	KM409696	0.000201	0.000201	0.000201	0.000206	1	0.000205	0.794059	0.76206	u	0.000457	0.999999
10	KM409697	0.072469	0.255976	0.003527	0.906681	0.000322	0.868942	0.016539	0.018859	0.000457	t	0.000302
11	KM409705	0.000201	0.000201	0.000201	0.000203	1	0.000203	0.577661	0.540893	0.999999	0.000302	u

Table B. 25: Descriptive statistics of *L. garvieae* isolates from Lesotho at different pH.

Descriptive Statistics_ pH (*L. garvieae*_ Lesotho isolates)											
Level	N	**pH 4.5**					**pH 5**				
Factor		Mean	Std.Dev.	Std.Err	-95,00%	95,00%	Mean	Std.Dev.	Std.Err	-95,00%	95,00%
Total	30	-0,006433	0,178165	0,032528	-0,072961	0,060095	0,538633	0,111092	0,020282	0,497151	0,580116
KM409665	3	0,158667	0,007506	0,004333	0,140022	0,177311	0,819667	0,026633	0,015377	0,753506	0,885827
KM409664	3	0,377667	0,006506	0,003756	0,361504	0,393829	0,637333	0,001528	0,000882	0,633539	0,641128
KM409668	3	-0,1	0	0	-0,1	-0,1	0,482	0,006	0,003464	0,467095	0,496905
KM409689	3	0,206	0,011358	0,006557	0,177786	0,234214	0,429333	0,006506	0,003756	0,413171	0,445496
KM409691	3	-0,133333	0,025166	0,01453	-0,195849	-0,070817	0,456667	0,002082	0,001202	0,451496	0,461838
KM409694	3	-0,093333	0,005774	0,003333	-0,107676	-0,078991	0,531333	0,005859	0,003383	0,516778	0,545889
KM409695	3	-0,14	0,04	0,023094	-0,239366	-0,040634	0,476333	0,003215	0,001856	0,468348	0,484319
KM409701	3	-0,106667	0,025166	0,01453	-0,169183	-0,044151	0,490667	0,004163	0,002404	0,480324	0,501009
KM409702	3	-0,11	0	0	-0,11	-0,11	0,560667	0,010214	0,005897	0,535293	0,586041
KM409703	3	-0,123333	0,015275	0,008819	-0,161279	-0,085388	0,502333	0,006351	0,003667	0,486557	0,51811
Level	N	**pH 7**					**pH 8**				
Factor		Mean	Std.Dev.	Std.Err	-95,00%	95,00%	Mean	Std.Dev.	Std.Err	-95,00%	95,00%
	30	1,3836	0,066552	0,012151	1,358749	1,408451	1,532467	0,080611	0,014717	1,502366	1,562567
KM409665	3	1,307333	0,020207	0,011667	1,257136	1,357531	1,332	0,010817	0,006245	1,30513	1,35887
KM409664	3	1,513333	0,018717	0,010806	1,466837	1,559829	1,647	0,010583	0,00611	1,62071	1,67329
KM409668	3	1,348667	0,005033	0,002906	1,336163	1,36117	1,492333	0,019502	0,01126	1,443887	1,540779
KM409689	3	1,294333	0,002517	0,001453	1,288082	1,300585	1,555667	0,005774	0,003333	1,541324	1,570009
KM409691	3	1,368	0,001732	0,001	1,363697	1,372303	1,519333	0,018148	0,010477	1,474252	1,564414
KM409694	3	1,411	0,0151	0,008718	1,37349	1,44851	1,551667	0,012583	0,007265	1,520409	1,582925
KM409695	3	1,326667	0,013317	0,007688	1,293586	1,359747	1,581667	0,02203	0,012719	1,52694	1,636393
KM409701	3	1,421667	0,005132	0,002963	1,408919	1,434414	1,559	0,01833	0,010583	1,513465	1,604535

KM409702	3	1,448333	0,005508	0,00318	1,434652	1,462015	1,573	0,019672	0,011358	1,524131	1,621869
KM409703	3	1,396667	0,012055	0,00696	1,366719	1,426614	1,513	0,013115	0,007572	1,480421	1,545579
Level	N	**pH 9.5**									
Factor		Mean	Std.Dev.	Std.Err	-95,00%	95,00%					
Total	30	1,294733	0,080551	0,014707	1,264655	1,324812					
KM409665	3	1,198333	0,033561	0,019376	1,114963	1,281703					
KM409664	3	1,448333	0,025541	0,014746	1,384886	1,51178					
KM409668	3	1,252667	0,013796	0,007965	1,218395	1,286938					
KM409689	3	1,343	0,015524	0,008963	1,304436	1,381564					
KM409691	3	1,362	0,028688	0,016563	1,290735	1,433265					
KM409694	3	1,239667	0,036665	0,021169	1,148585	1,330748					
KM409695	3	1,306	0,052431	0,030271	1,175754	1,436246					
KM409701	3	1,265	0,035	0,020207	1,178055	1,351945					
KM409702	3	1,214333	0,071361	0,0412	1,037064	1,391603					
KM409703	3	1,318	0,042438	0,024502	1,212578	1,423422					

Table B. 26: Tukey HSD analysis for *L. garvieae* isolates from Lesotho at different pH 4.5.

Cell No.	**Tukey HSD test; variable pH 4.5** (Spreadsheet16 in Workbook3) (Approximate Probabilities for Post Hoc Tests) Error: Between MS = ,00034, df = 20,000										
	isolates	KM409665	KM409664	KM409668	KM409689	KM409691	KM409694	KM409695	KM409701	KM409702	KM409703
		0.15867	0.37767	-0.1	0.206	-0.1333	-0.0933	-0.14	-0.1067	-0.11	-0.1233
1	KM409665	a	0.000179	0.000179	0.105255	0.000179	0.000179	0.000179	0.000179	0.000179	0.000179
2	KM409664	0.000179	b	0.000179	0.000179	0.000179	0.000179	0.000179	0.000179	0.000179	0.000179
3	KM409668	0.000179	0.000179	c	0.000179	0.471574	0.999981	0.249005	0.999981	0.999454	0.852267
4	KM409689	0.105255	0.000179	0.000179	a	0.000179	0.000179	0.000179	0.000179	0.000179	0.000179
5	KM409691	0.000179	0.000179	0.471574	0.000179	c	0.249005	0.999981	0.738742	0.852267	0.999454
6	KM409694	0.000179	0.000179	0.999981	0.000179	0.249005	c	0.114423	0.995071	0.977358	0.605951
7	KM409695	0.000179	0.000179	0.249005	0.000179	0.999981	0.114423	c	0.471574	0.605951	0.977358
8	KM409701	0.000179	0.000179	0.999981	0.000179	0.738742	0.995071	0.471574	c	1	0.977358
9	KM409702	0.000179	0.000179	0.999454	0.000179	0.852267	0.977358	0.605951	1	c	0.995071
10	KM409703	0.000179	0.000179	0.852267	0.000179	0.999454	0.605951	0.977358	0.977358	0.995071	c

Table B. 27: Tukey HSD analysis for *L. garvieae* isolates from Lesotho at different pH 5.

Cell No.	**Tukey HSD test; variable pH 5** (Spreadsheet16 in Workbook3) (Approximate Probabilities for Post Hoc Tests) Error: Between MS = ,00010, df = 20,000										
	isolates	KM409665	KM409664	KM409668	KM409689	KM409691	KM409694	KM409695	KM409701	KM409702	KM409703
		0.81967	0.63733	0.482	0.42933	0.45667	0.53133	0.47633	0.49067	0.56067	0.50233
1	KM409665	d	0.000179	0.000179	0.000179	0.000179	0.000179	0.000179	0.000179	0.000179	0.000179
2	KM409664	0.000179	e	0.000179	0.000179	0.000179	0.000179	0.000179	0.000179	0.000179	0.000179
3	KM409668	0.000179	0.000179	f	0.000256	0.117983	0.000375	0.999265	0.983457	0.000179	0.330275
4	KM409689	0.000179	0.000179	0.000256	g	0.073907	0.000179	0.000558	0.00019	0.000179	0.000179
5	KM409691	0.000179	0.000179	0.117983	0.073907	f g	0.000179	0.371323	0.013657	0.000179	0.00073
6	KM409694	0.000179	0.000179	0.000375	0.000179	0.000179	h	0.00022	0.0024	0.045257	0.049168
7	KM409695	0.000179	0.000179	0.999265	0.000558	0.371323	0.00022	f	0.753817	0.000179	0.101232
8	KM409701	0.000179	0.000179	0.983457	0.00019	0.013657	0.0024	0.753817	f	0.000179	0.903984
9	KM409702	0.000179	0.000179	0.000179	0.000179	0.000179	0.045257	0.000179	0.000179	i	0.000198
10	KM409703	0.000179	0.000179	0.330275	0.000179	0.00073	0.049168	0.101232	0.903984	0.000198	f

Table B. 28: Tukey HSD analysis for *L. garvieae* isolates from Lesotho at different pH 7.

Cell No.	**Tukey HSD test; variable pH 7** (Approximate Probabilities for Post Hoc Tests); Error: Between MS = ,00014, df = 20,000										
	isolates	KM409665	KM409664	KM409668	KM409689	KM409691	KM409694	KM409695	KM409701	KM409702	KM409703
		1.3073	1.5133	1.3487	1.2943	1.368	1.411	1.3267	1.4217	1.4483	1.3967
1	KM409665	j	0.000179	0.01067	0.930175	0.000292	0.000179	0.608066	0.000179	0.000179	0.000179
2	KM409664	0.000179	k	0.000179	0.000179	0.000179	0.000179	0.000179	0.000179	0.000221	0.000179
3	KM409668	0.01067	0.000179	l	0.000691	0.608066	0.000256	0.4427	0.00019	0.000179	0.002454
4	KM409689	0.930175	0.000179	0.000691	j	0.000189	0.000179	0.073898	0.000179	0.000179	0.000179
5	KM409691	0.000292	0.000179	0.608066	0.000189	l m	0.007367	0.01067	0.000778	0.000186	0.150807
6	KM409694	0.000179	0.000179	0.000256	0.000179	0.007367	n	0.000179	0.978576	0.025736	0.88368
7	KM409695	0.608066	0.000179	0.4427	0.073898	0.01067	0.000179	j l	0.000179	0.000179	0.000195
8	KM409701	0.000179	0.000179	0.00019	0.000179	0.000778	0.978576	0.000179	n m	0.215806	0.284773
9	KM409702	0.000179	0.000221	0.000179	0.000179	0.000186	0.025736	0.000179	0.215806	m	0.001143
10	KM409703	0.000179	0.000179	0.002454	0.000179	0.150807	0.88368	0.000195	0.284773	0.001143	m n

Table B. 29: Tukey HSD analysis for *L. garvieae* isolates from Lesotho at different pH 8.

Cell No.	**Tukey HSD test; variable pH 8** (Approximate Probabilities for Post Hoc Tests) Error: Between MS = ,00025, df = 20,000										
	isolates	KM409665	KM409664	KM409668	KM409689	KM409691	KM409694	KM409695	KM409701	KM409702	KM409703
		1.332	1.647	1.4923	1.5557	1.5193	1.5517	1.5817	1.559	1.573	1.513
1	KM409665	o	0.000179	0.000179	0.000179	0.000179	0.000179	0.000179	0.000179	0.000179	0.000179
2	KM409664	0.000179	p	0.000179	0.000201	0.000179	0.000192	0.002071	0.000215	0.000588	0.000179
3	KM409668	0.000179	0.000179	q	0.002851	0.555264	0.0055	0.000208	0.001675	0.000304	0.83466
4	KM409689	0.000179	0.000201	0.002851	r	0.198812	0.999999	0.602577	1	0.931751	0.081167
5	KM409691	0.000179	0.000179	0.555264	0.198812	q r	0.325383	0.003362	0.125894	0.014068	0.999957
6	KM409694	0.000179	0.000192	0.0055	0.999999	0.325383	r	0.419005	0.999855	0.809687	0.144922
7	KM409695	0.000179	0.002071	0.000208	0.602577	0.003362	0.419005	r	0.755242	0.999442	0.001232
8	KM409701	0.000179	0.000215	0.001675	1	0.125894	0.999855	0.755242	r	0.981171	0.048615
9	KM409702	0.000179	0.000588	0.000304	0.931751	0.014068	0.809687	0.999442	0.981171	r	0.004925
10	KM409703	0.000179	0.000179	0.83466	0.081167	0.999957	0.144922	0.001232	0.048615	0.004925	q r

Table B. 30: Tukey HSD analysis for *L. garvieae* isolates from Lesotho at different pH 9.5.

Cell No.	**Tukey HSD test; variable pH 9.5** (Approximate Probabilities for Post Hoc Tests) Error: Between MS = ,00152, df = 20,000										
	isolates	KM409665	KM409664	KM409668	KM409689	KM409691	KM409694	KM409695	KM409701	KM409702	KM409703
		1.1983	1.4483	1.2527	1.343	1.362	1.2397	1.306	1.265	1.2143	1.318
1	KM409665	s	0.000187	0.781117	0.006098	0.001744	0.943197	0.069469	0.552678	0.999947	0.03236
2	KM409664	0.000187	t	0.000336	0.080199	0.234942	0.000241	0.007137	0.00056	0.000193	0.015991
3	KM409668	0.781117	0.000336	s u	0.190454	0.062631	0.999991	0.797512	0.999994	0.963477	0.57824
4	KM409689	0.006098	0.080199	0.190454	t u	0.999781	0.090531	0.970596	0.351226	0.017873	0.99809
5	KM409691	0.001744	0.234942	0.062631	0.999781	t u	0.027182	0.752695	0.131401	0.004985	0.919777
6	KM409694	0.943197	0.000241	0.999991	0.090531	0.027182	s u	0.55906	0.99789	0.99789	0.345983
7	KM409695	0.069469	0.007137	0.797512	0.970596	0.752695	0.55906	s u	0.945757	0.177201	0.999995
8	KM409701	0.552678	0.00056	0.999994	0.351226	0.131401	0.99789	0.945757	s u	0.838443	0.802857
9	KM409702	0.999947	0.000193	0.963477	0.017873	0.004985	0.99789	0.177201	0.838443	s u	0.088731
10	KM409703	0.03236	0.015991	0.57824	0.99809	0.919777	0.345983	0.999995	0.802857	0.088731	u

Table B. 31: Descriptive statistics of the *Weissella* species at different pH.

Descriptive Statistics_ pH (*Weissella* species)											
Level	N	**pH 4.5**					**pH 5**				
Factor		Mean	Std.Dev.	Std.Err	-95,00%	95,00%	Mean	Std.Dev.	Std.Err	-95,00%	95,00%
Total	6	-0,0975	0,0499	0,02037	-0,1499	-0,0451	0,18183	0,05326	0,02174	0,12594	0,237726
KM409656	3	-0,1143	0,00737	0,00426	-0,1326	-0,096	0,187	0,01082	0,00625	0,16013	0,21387
KM409657	3	-0,0807	0,07295	0,04212	-0,2619	0,10055	0,17667	0,08303	0,04794	-0,0296	0,38293
Level	N	**pH 7**					**pH 8**				
Factor		Mean	Std.Dev.	Std.Err	-95,00%	95,00%	Mean	Std.Dev.	Std.Err	-95,00%	95,00%
Total	6	0,35933	0,02743	0,0112	0,33055	0,38812	0,2135	0,09379	0,03829	0,11507	0,311927
KM409656	3	0,33933	0,02173	0,01255	0,28535	0,39332	0,298	0,02066	0,01193	0,24667	0,349332
KM409657	3	0,37933	0,01443	0,00833	0,34348	0,41519	0,129	0,012	0,00693	0,09919	0,15881
Level	N	**pH 9.5**									
Factor		Mean	Std.Dev.	Std.Err	-95,00%	95,00%					
Total	6	0,28167	0,04078	0,01665	0,23888	0,32446					
KM409656	3	0,26467	0,03383	0,01953	0,18063	0,3487					
KM409657	3	0,29867	0,04632	0,02674	0,18361	0,41373					

Table B. 32: Tukey HSD analysis for *Weissella* species at different pH.

Cell No.	Tukey HSD test; variable pH 4.5 Approximate Probabilities for Post Hoc Tests Error: Between MS = ,00269, df = 4,0000		
	isolates	KM409656	KM409657
		-0,1143	-0,0807
1	KM409656	a	0,471189
2	KM409657	0,471189	a

Cell No.	Tukey HSD test; variable pH 5 Approximate Probabilities for Post Hoc Tests Error: Between MS = ,00351, df = 4,0000		
	isolates	KM409656	KM409657
		0,187	0,17667
1	KM409656	b	0,841357
2	KM409657	0,841357	b

Cell No.	Tukey HSD test; variable pH 7 Approximate Probabilities for Post Hoc Tests Error: Between MS = ,00034, df = 4,0000		
	isolates	KM409656	KM409657
1	KM409656	c	0,056831
2	KM409657	0,056831	c

Cell No.	Tukey HSD test; variable pH 8 Approximate Probabilities for Post Hoc Tests Error: Between MS = ,00029, df = 4,0000		
	isolates	KM409656	KM409657
1	KM409656	d	0,000479
2	KM409657	0,000479	e

Cell No.	Tukey HSD test; variable pH 9.5 Approximate Probabilities for Post Hoc Tests Error: Between MS = ,00164, df = 4,0000		
	isolates	KM409656	KM409657
		0,26467	0,29867
1	KM409656	f	0,362774
2	KM409657	0,362774	f

Table B. 33: Descriptive statistics of *C. maltaromaticum* sp. at different pH.

Descriptive Statistics_ *C. maltaromaticum*											
Level of Factor	N	**pH 4.5**					**pH 5**				
		Mean	Std.Dev.	Std.Err	-95,00%	95,00%	Mean	Std.Dev.	Std.Err	-95,00%	95,00%
Total	9	-0,130556	0,039772	0,013257	-0,161127	-0,099984	0,877333	0,053642	0,017881	0,8361	0,918566
KM409658	3	-0,098333	0,01115	0,006438	-0,126033	-0,070634	0,905333	0,065623	0,037887	0,742318	1,068349
KM409660	3	-0,18	0,017321	0,01	-0,223027	-0,136973	0,899	0,006083	0,003512	0,88389	0,91411
KM409659	3	-0,113333	0,015275	0,008819	-0,151279	-0,075388	0,827667	0,039829	0,022995	0,728726	0,926607
Level of Factor	N	**pH 7**					**pH 8**				
		Mean	Std.Dev.	Std.Err	-95,00%	95,00%	Mean	Std.Dev.	Std.Err	-95,00%	95,00%
Total	9	1,314333	0,061384	0,020461	1,267149	1,361517	1,298444	0,063865	0,021288	1,249353	1,347536
KM409658	3	1,305	0,00781	0,004509	1,285598	1,324402	1,247	0,023	0,013279	1,189865	1,304135
KM409660	3	1,250333	0,025166	0,01453	1,187817	1,312849	1,269667	0,022811	0,01317	1,213001	1,326332
KM409659	3	1,387667	0,006028	0,00348	1,372693	1,40264	1,378667	0,020008	0,011552	1,328963	1,42837
Level of Factor	N	**pH 9.5**									
		Mean	Std.Dev.	Std.Err	-95,00%	95,00%					
Total	9	1,468222	0,030548	0,010183	1,444741	1,491704					
KM409658	3	1,458	0,026907	0,015535	1,391159	1,524841					
KM409660	3	1,487333	0,044736	0,025828	1,376202	1,598464					
KM409659	3	1,459333	0,013577	0,007839	1,425606	1,49306					

Table B. 34: Tukey HSD analysis for *C. maltaromaticum* sp. at different pH.

Cell No.	Tukey HSD test; variable pH 4.5 Approximate Probabilities for Post Hoc Tests Error: Between MS = ,00022, df = 6,0000			
	isolates	KM409658	KM409660	KM409659
		-0,0983	-0,18	-0,1133
1	KM409658	a	0,00143	0,474767
2	KM409660	0,00143	b	0,003755
3	KM409659	0,474767	0,003755	a

Cell No.	Tukey HSD test; variable pH 5 Approximate Probabilities for Post Hoc Tests Error: Between MS = ,00198, df = 6,0000			
	isolates	KM409658	KM409660	KM409659
		0,90533	0,899	0,82767
1	KM409658	c	0,983505	0,161798
2	KM409660	0,983505	c	0,201742
3	KM409659	0,161798	0,201742	c

Cell No.	Tukey HSD test; variable pH 7 Approximate Probabilities for Post Hoc Tests Error: Between MS = ,00024, df = 6,0000			
	isolates	KM409658	KM409660	KM409659
		1,305	1,2503	1,3877
1	KM409658	d	0,012324	0,001735
2	KM409660	0,012324	e	0,000283
3	KM409659	0,001735	0,000283	f

Cell No.	Tukey HSD test; variable pH 8 Approximate Probabilities for Post Hoc Tests Error: Between MS = ,00048, df = 6,0000			
	isolates	KM409658	KM409660	KM409659
		1,247	1,2697	1,3787
1	KM409658	g	0,463727	0,000978
2	KM409660	0,463727	g	0,002364
3	KM409659	0,000978	0,002364	h

Cell No.	Tukey HSD test; variable pH 9.5 Approximate Probabilities for Post Hoc Tests Error: Between MS = ,00097, df = 6,0000			
	isolates	KM409658	KM409660	KM409659
		1,458	1,4873	1,4593
1	KM409658	i	0,51977	0,998554
2	KM409660	0,51977	i	0,547824
3	KM409659	0,998554	0,547824	i

Statistical Analysis Results: Salinity

Table B. 35: Descriptive statistics of *L. garvieae* isolates from South Africa at different salt (NaCl) concentrations.

Descriptive Statistics_ Salinity (*L. garvieae*_ South African isolates)											
Level Factor	N	**0%**					**3.5 %**				
		Mean	Std.Dev.	Std.Err	-95,00%	95,00%	Mean	Std.Dev.	Std.Err	-95,00%	95,00%
Total	33	0,280939	0,087267	0,015191	0,249996	0,311883	0,187394	0,094226	0,016403	0,153983	0,220805
KM409663	3	0,308667	0,009452	0,005457	0,285188	0,332146	0,308	0,01179	0,006807	0,278712	0,337288
KM409669	3	0,331	0,006245	0,003606	0,315487	0,346513	0,313667	0,009713	0,005608	0,289539	0,337794
KM409673	3	0,281667	0,048521	0,028014	0,161133	0,402201	0,2	0,018682	0,010786	0,153592	0,246408
KM409675	3	0,360333	0,011015	0,00636	0,33297	0,387696	0,205	0,015875	0,009165	0,165566	0,244434
KM409680	3	0,288333	0,002082	0,001202	0,283162	0,293504	0,203667	0,007371	0,004256	0,185356	0,221978
KM409682	3	0,268	0,027404	0,015822	0,199924	0,336076	0,294333	0,011719	0,006766	0,265222	0,323445
KM409685	3	0,025	0,014933	0,008622	-0,012096	0,062096	0,127	0,020421	0,01179	0,076272	0,177728
KM659864	3	0,310667	0,019088	0,01102	0,263251	0,358083	0,003667	0,004619	0,002667	-0,007807	0,01514
KM409696	3	0,305	0,00781	0,004509	0,285598	0,324402	0,162	0,011358	0,006557	0,133786	0,190214
KM409697	3	0,317	0,00781	0,004509	0,297598	0,336402	0,077667	0,01115	0,006438	0,049967	0,105366
KM409705	3	0,294667	0,019858	0,011465	0,245337	0,343996	0,166333	0,023502	0,013569	0,107952	0,224715
Level Factor	N	**5%**					**6.5 %**				
		Mean	Std.Dev.	Std.Err	-95,00%	95,00%	Mean	Std.Dev.	Std.Err	-95,00%	95,00%
Total	33	0,090606	0,070054	0,012195	0,065766	0,115446	0,053485	0,055725	0,009701	0,033725	0,073244
KM409663	3	0,124	0,013748	0,007937	0,089849	0,158151	0,074667	0,028537	0,016476	0,003778	0,145555
KM409669	3	-0,015	0,018248	0,010536	-0,060331	0,030331	0,005667	0,016653	0,009615	-0,035702	0,047036
KM409673	3	0,194333	0,016289	0,009404	0,153869	0,234798	0,013	0,037987	0,021932	-0,081365	0,107365
KM409675	3	0,156667	0,010693	0,006173	0,130105	0,183229	0,137	0,023643	0,01365	0,078267	0,195733

KM409680	3	0,120333	0,013429	0,007753	0,086974	0,153692	0,08	0,007211	0,004163	0,062087	0,097913
KM409682	3	0,099	0,00781	0,004509	0,079598	0,118402	0,129333	0,016653	0,009615	0,087964	0,170702
KM409685	3	-0,003333	0,003512	0,002028	-0,012057	0,005391	-0,022333	0,00611	0,003528	-0,037512	-0,007155
KM659864	3	-0,018	0,006928	0,004	-0,035211	-0,000789	-0,011	0,002646	0,001528	-0,017572	-0,004428
KM409696	3	0,117	0,018682	0,010786	0,070592	0,163408	0,093333	0,010408	0,006009	0,067478	0,119189
KM409697	3	0,119667	0,031501	0,018187	0,041413	0,19792	0,022333	0,007506	0,004333	0,003689	0,040978
KM409705	3	0,102	0,004583	0,002646	0,090616	0,113384	0,066333	0,020008	0,011552	0,01663	0,116037

Table B. 36: Tukey HSD analysis for *L. garvieae* isolates from South Africa at 0 % NaCl.

Cell No.	**Tukey HSD test; variable NaCl 0 %** (Approximate Probabilities for Post Hoc Tests); Error: Between MS = ,00041, df = 22,000											
	isolates	KM409663	KM409669	KM409673	KM409675	KM409680	KM409682	KM409685	KM659864	KM409696	KM409697	KM409705
		0.30867	0.331	0.28167	0.36033	0.28833	0.268	0.025	0.31067	0.305	0.317	0.29467
1	KM409663	a	0.946491	0.848001	0.11937	0.970496	0.369672	0.000201	1	1	0.999981	0.998192
2	KM409669	0.946491	a b	0.15555	0.777285	0.308654	0.029053	0.000201	0.970496	0.874218	0.998192	0.521516
3	KM409673	0.848001	0.15555	a b c	0.003509	0.999998	0.998529	0.000201	0.788119	0.930545	0.558966	0.99903
4	KM409675	0.11937	0.777285	0.003509	a b	0.008668	0.000649	0.000201	0.149883	0.077135	0.289705	0.020405
5	KM409680	0.970496	0.308654	0.999998	0.008668	a b c	0.970496	0.000201	0.946491	0.99281	0.798732	0.999999
6	KM409682	0.369672	0.029053	0.998529	0.000649	0.970496	a c	0.000201	0.308654	0.496895	0.161392	0.857032
7	KM409685	0.000201	0.000201	0.000201	0.000201	0.000201	0.000201	d	0.000201	0.000201	0.000201	0.000201
8	KM659864	1	0.970496	0.788119	0.149883	0.946491	0.308654	0.000201	a b c	1	0.999999	0.994741
9	KM409696	1	0.874218	0.930545	0.077135	0.99281	0.496895	0.000201	1	a b c	0.999509	0.999868
10	KM409697	0.999981	0.998192	0.558966	0.289705	0.798732	0.161392	0.000201	0.999999	0.999509	a b c	0.946491
11	KM409705	0.998192	0.521516	0.99903	0.020405	0.999999	0.857032	0.000201	0.994741	0.999868	0.946491	a b c

Table B. 37: Tukey HSD analysis for *L. garvieae* isolates from South Africa at 3.5 % NaCl.

Cell No.	**Tukey HSD test; variable NaCl 3.5 %** (Approximate Probabilities for Post Hoc Tests) ; Error: Between MS = ,00021, df = 22,000											
	isolates	KM409663	KM409669	KM409673	KM409675	KM409680	KM409682	KM409685	KM659864	KM409696	KM409697	KM409705
		0.308	0.31367	0.2	0.205	0.20367	0.29433	0.127	0.00367	0.162	0.07767	0.16633
1	KM409663	e	0.999988	0.000201	0.000201	0.000201	0.980247	0.000201	0.000201	0.000201	0.000201	0.000201
2	KM409669	0.999988	e	0.000201	0.000201	0.000201	0.844996	0.000201	0.000201	0.000201	0.000201	0.000201
3	KM409673	0.000201	0.000201	f	0.999996	1	0.000202	0.000298	0.000201	0.098758	0.000201	0.196016
4	KM409675	0.000201	0.000201	0.999996	f	1	0.000203	0.000233	0.000201	0.041323	0.000201	0.088316
5	KM409680	0.000201	0.000201	1	1	f	0.000203	0.000244	0.000201	0.052469	0.000201	0.110269
6	KM409682	0.980247	0.844996	0.000202	0.000203	0.000203	g	0.000201	0.000201	0.000201	0.000201	0.000201
7	KM409685	0.000201	0.000201	0.000298	0.000233	0.000244	0.000201	h	0.000201	0.160046	0.012728	0.07886
8	KM659864	0.000201	0.000201	0.000201	0.000201	0.000201	0.000201	0.000201	i	0.000201	0.000279	0.000201
9	KM409696	0.000201	0.000201	0.098758	0.041323	0.052469	0.000201	0.160046	0.000201	f h	0.000209	0.999999
10	KM409697	0.000201	0.000201	0.000201	0.000201	0.000201	0.000201	0.012728	0.000279	0.000209	j	0.000204
11	KM409705	0.000201	0.000201	0.196016	0.088316	0.110269	0.000201	0.07886	0.000201	0.999999	0.000204	f h

Table B. 38: Tukey HSD analysis for *L. garvieae* isolates from South Africa at 5 % NaCl.

Cell No.	**Tukey HSD test; variable NaCl 5 %** (Approximate Probabilities for Post Hoc Tests); Error: Between MS = ,00023, df = 22,000											
	isolates	KM409663	KM409669	KM409673	KM409675	KM409680	KM409682	KM409685	KM659864	KM409696	KM409697	KM409705
		0.124	-0.015	0.19433	0.15667	0.12033	0.099	-0.0033	-0.018	0.117	0.11967	0.102
1	KM409663	k	0.000201	0.000621	0.296649	1	0.647955	0.000201	0.000201	0.999952	0.999999	0.787608
2	KM409669	0.000201	l	0.000201	0.000201	0.000201	0.000201	0.996126	1	0.000201	0.000201	0.000201
3	KM409673	0.000621	0.000201	m	0.149681	0.000403	0.000203	0.000201	0.000201	0.000304	0.000378	0.000205
4	KM409675	0.296649	0.000201	0.149681	k m	0.181628	0.004993	0.000201	0.000201	0.110671	0.164993	0.008551
5	KM409680	1	0.000201	0.000403	0.181628	k	0.815106	0.000201	0.000201	1	1	0.914857
6	KM409682	0.647955	0.000201	0.000203	0.004993	0.815106	k	0.000202	0.000201	0.92326	0.840832	1
7	KM409685	0.000201	0.996126	0.000201	0.000201	0.000201	0.000202	l	0.978924	0.000201	0.000201	0.000202
8	KM659864	0.000201	1	0.000201	0.000201	0.000201	0.000201	0.978924	l n	0.000201	0.000201	0.000201
9	KM409696	0.999952	0.000201	0.000304	0.110671	1	0.92326	0.000201	0.000201	k	1	0.975431
10	KM409697	0.999999	0.000201	0.000378	0.164993	1	0.840832	0.000201	0.000201	1	k	0.931109
11	KM409705	0.787608	0.000201	0.000205	0.008551	0.914857	1	0.000202	0.000201	0.975431	0.931109	k

Table B. 39: Tukey HSD analysis for *L. garvieae* isolates from South Africa at 6.5 % NaCl.

Cell No.	**Tukey HSD test; variable Nacl 6.5 %** (Approximate Probabilities for Post Hoc Tests); Error: Between MS = ,00037, df = 22,000											
	isolates	KM409663	KM409669	KM409673	KM409675	KM409680	KM409682	KM409685	KM659864	KM409696	KM409697	KM409705
		0.07467	0.00567	0.013	0.137	0.08	0.12933	-0.0223	-0.011	0.09333	0.02233	0.06633
1	KM409663	o	0.007999	0.02267	0.020649	1	0.058858	0.000303	0.000827	0.976626	0.079776	0.99997
2	KM409669	0.007999	p	0.999991	0.000202	0.00374	0.000202	0.7732	0.989504	0.000656	0.989504	0.02606
3	KM409673	0.02267	0.999991	p q	0.000202	0.01065	0.000205	0.490587	0.892111	0.001629	0.999917	0.070089
4	KM409675	0.020649	0.000202	0.000202	r	0.043096	0.999986	0.000201	0.000201	0.223721	0.000206	0.006303
5	KM409680	1	0.00374	0.01065	0.043096	o	0.116167	0.000243	0.000469	0.998163	0.039374	0.997752
6	KM409682	0.058858	0.000202	0.000205	0.999986	0.116167	o r	0.000201	0.000201	0.465037	0.00022	0.018803
7	KM409685	0.000303	0.7732	0.490587	0.000201	0.000243	0.000201	p q	0.999535	0.000205	0.200518	0.00059
8	KM659864	0.000827	0.989504	0.892111	0.000201	0.000469	0.000201	0.999535	p q	0.000231	0.568956	0.002451
9	KM409696	0.976626	0.000656	0.001629	0.223721	0.998163	0.465037	0.000205	0.000231	o r	0.00601	0.806885
10	KM409697	0.079776	0.989504	0.999917	0.000206	0.039374	0.00022	0.200518	0.568956	0.00601	o p q	0.215769
11	KM409705	0.99997	0.02606	0.070089	0.006303	0.997752	0.018803	0.00059	0.002451	0.806885	0.215769	o q

Table B. 40: Descriptive statistics of *L. garvieae* isolates from Lesotho at different salt concentrations.

Descriptive Statistics_ Temperature (*L. garvieae*_ Lesotho isolates)											
Level	N	**0%**					**3.5 %**				
Factor		Mean	Std.Dev.	Std.Err	-95,00%	95,00%	Mean	Std.Dev.	Std.Err	-95,00%	95,00%
Total	30	0,296167	0,032463	0,005927	0,284045	0,308289	0,1687	0,044772	0,008174	0,151982	0,185418
KM409665	3	0,328667	0,009609	0,005548	0,304797	0,352537	0,155	0,014799	0,008544	0,118238	0,191762
KM409664	3	0,279	0,074357	0,04293	0,094286	0,463714	0,129667	0,009609	0,005548	0,105797	0,153537
KM409668	3	0,285333	0,016258	0,009387	0,244945	0,325721	0,181667	0,003055	0,001764	0,174078	0,189256
KM409689	3	0,308	0,011269	0,006506	0,280005	0,335995	0,204667	0,017156	0,009905	0,162048	0,247285
KM409691	3	0,286333	0,014224	0,008212	0,250998	0,321669	0,158667	0,053594	0,030943	0,025531	0,291802
KM409694	3	0,318667	0,014572	0,008413	0,282469	0,354865	0,122	0,014731	0,008505	0,085406	0,158594
KM409695	3	0,280667	0,030039	0,017343	0,206046	0,355287	0,165	0,027622	0,015948	0,096382	0,233618
KM409701	3	0,309667	0,000577	0,000333	0,308232	0,311101	0,143	0,031	0,017898	0,065992	0,220008
KM409702	3	0,315333	0,007506	0,004333	0,296689	0,333978	0,26	0,04392	0,025357	0,150896	0,369104
KM409703	3	0,25	0,014	0,008083	0,215222	0,284778	0,167333	0,003055	0,001764	0,159744	0,174922
Level	N	**5%**					**6.5 %**				
Factor		Mean	Std.Dev.	Std.Err	-95,00%	95,00%	Mean	Std.Dev.	Std.Err	-95,00%	95,00%
Total	30	0,042667	0,048644	0,008881	0,024503	0,060831	0,0065	0,01636	0,002987	0,000391	0,012609
KM409665	3	0,027667	0,008021	0,004631	0,007742	0,047591	0,033667	0,003512	0,002028	0,024943	0,042391
KM409664	3	0,022667	0,012858	0,007424	-0,009275	0,054608	0,028	0,006	0,003464	0,013095	0,042905
KM409668	3	0,047667	0,017039	0,009838	0,005339	0,089994	0	0,002646	0,001528	-0,006572	0,006572
KM409689	3	0,007667	0,005033	0,002906	-0,004837	0,02017	0,005667	0,005033	0,002906	-0,006837	0,01817
KM409691	3	0,139	0,01249	0,007211	0,107973	0,170027	-0,019	0,013454	0,007767	-0,052421	0,014421
KM409694	3	0,007333	0,004726	0,002728	-0,004406	0,019073	0,015333	0,009292	0,005364	-0,007748	0,038415
KM409695	3	0,048667	0,010066	0,005812	0,02366	0,073673	-0,008	0,009539	0,005508	-0,031697	0,015697
KM409701	3	0,109667	0,05862	0,033844	-0,035954	0,255287	0,001667	0,001155	0,000667	-0,001202	0,004535

KM409702	3	-0,009	0,013748	0,007937	-0,043151	0,025151	0,006667	0,002887	0,001667	-0,000504	0,013838
KM409703	3	0,025333	0,003512	0,002028	0,016609	0,034057	0,001	0,008888	0,005132	-0,021079	0,023079

Table B. 41: Tukey HSD analysis for *L. garvieae* isolates from Lesotho at 0 % NaCl.

Cell No.	**Tukey HSD test; variable 0 %** (Approximate Probabilities for Post Hoc Tests); Error: Between MS = ,00076, df = 20,000										
	isolates	KM409665	KM409664	KM409668	KM409689	KM409691	KM409694	KM409695	KM409701	KM409702	KM409703
		0.32867	0.279	0.28533	0.308	0.28633	0.31867	0.28067	0.30967	0.31533	0.25
1	KM409665	a	0.481977	0.652801	0.993815	0.679578	0.999981	0.526153	0.99665	0.99979	0.05451
2	KM409664	0.481977	a	1	0.944843	0.999999	0.74841	1	0.924724	0.825836	0.944843
3	KM409668	0.652801	1	a	0.988239	1	0.883949	1	0.981153	0.933267	0.846563
4	KM409689	0.993815	0.944843	0.988239	a	0.991377	0.999967	0.960981	1	0.999999	0.28805
5	KM409691	0.679578	0.999999	1	0.991377	a	0.900486	1	0.985707	0.944843	0.825836
6	KM409694	0.999981	0.74841	0.883949	0.999967	0.900486	a	0.788589	0.999992	1	0.128673
7	KM409695	0.526153	1	1	0.960981	1	0.788589	a	0.944843	0.859644	0.924724
8	KM409701	0.99665	0.924724	0.981153	1	0.985707	0.999992	0.944843	a	1	0.256436
9	KM409702	0.99979	0.825836	0.933267	0.999999	0.944843	1	0.859644	1	a	0.167939
10	KM409703	0.05451	0.944843	0.846563	0.28805	0.825836	0.128673	0.924724	0.256436	0.167939	a

Table B. 42: Tukey HSD analysis for *L. garvieae* isolates from Lesotho at 3.5 % NaCl.

Cell No.	**Tukey HSD test; variable 3.5 %** (Approximate Probabilities for Post Hoc Tests); Error: Between MS = ,00074, df = 20,000										
	isolates	KM409665	KM409664	KM409668	KM409689	KM409691	KM409694	KM409695	KM409701	KM409702	KM409703
		0.155	0.12967	0.18167	0.20467	0.15867	0.122	0.165	0.143	0.26	0.16733
1	KM409665	b	0.973248	0.963316	0.463093	1	0.881409	0.999978	0.9999	0.003979	0.999875
2	KM409664	0.973248	b	0.403817	0.068512	0.940137	0.999998	0.836083	0.999764	0.000461	0.783581
3	KM409668	0.963316	0.403817	b c	0.985704	0.985704	0.241015	0.998624	0.759225	0.050711	0.999578
4	KM409689	0.463093	0.068512	0.985704	b d	0.562128	0.03398	0.733944	0.207638	0.326503	0.79147
5	KM409691	1	0.940137	0.985704	0.562128	b d	0.806877	1	0.999147	0.005662	0.999994
6	KM409694	0.881409	0.999998	0.241015	0.03398	0.806877	b	0.644931	0.992331	0.000306	0.580531
7	KM409695	0.999978	0.836083	0.998624	0.733944	1	0.644931	b d	0.989411	0.010457	1
8	KM409701	0.9999	0.999764	0.759225	0.207638	0.999147	0.992331	0.989411	b d	0.001305	0.979305
9	KM409702	0.003979	0.000461	0.050711	0.326503	0.005662	0.000306	0.010457	0.001305	c d	0.013101
10	KM409703	0.999875	0.783581	0.999578	0.79147	0.999994	0.580531	1	0.979305	0.013101	b d

Table B. 43: Tukey HSD analysis for *L. garvieae* isolates from Lesotho at 5 % NaCl.

Cell No.	**Tukey HSD test; variable 5 %** (Approximate Probabilities for Post Hoc Tests); Error: Between MS = ,00045, df = 20,000										
	isolates	KM409665	KM409664	KM409668	KM409689	KM409691	KM409694	KM409695	KM409701	KM409702	KM409703
		0.02767	0.02267	0.04767	0.00767	0.139	0.00733	0.04867	0.10967	-0.009	0.02533
1	KM409665	e	1	0.970767	0.970767	0.000255	0.967626	0.960641	0.003847	0.531679	1
2	KM409664	1	e	0.896431	0.995856	0.000219	0.995141	0.8741	0.002097	0.707383	1
3	KM409668	0.970767	0.896431	e	0.418898	0.001266	0.408237	1	0.044915	0.083175	0.94363
4	KM409689	0.970767	0.995856	0.418898	e	0.000189	1	0.387358	0.000441	0.991216	0.986914
5	KM409691	0.000255	0.000219	0.001266	0.000189	f	0.000189	0.001417	0.783079	0.000179	0.000235
6	KM409694	0.967626	0.995141	0.408237	1	0.000189	e	0.377134	0.00043	0.992369	0.985172
7	KM409695	0.960641	0.8741	1	0.387358	0.001417	0.377134	e	0.050527	0.074295	0.928028
8	KM409701	0.003847	0.002097	0.044915	0.000441	0.783079	0.00043	0.050527	f	0.00021	0.002887
9	KM409702	0.531679	0.707383	0.083175	0.991216	0.000179	0.992369	0.074295	0.00021	e	0.614314
10	KM409703	1	1	0.94363	0.986914	0.000235	0.985172	0.928028	0.002887	0.614314	e

Table B. 44: Tukey HSD analysis for *L. garvieae* isolates from Lesotho at 6.5 % NaCl.

Cell No.	**Tukey HSD test; variable 6.5 %** (Approximate Probabilities for Post Hoc Tests); Error: Between MS = ,00005, df = 20,000										
	isolates	KM409665	KM409664	KM409668	KM409689	KM409691	KM409694	KM409695	KM409701	KM409702	KM409703
		0.03367	0.028	0	0.00567	-0.019	0.01533	-0.008	0.00167	0.00667	0.001
1	KM409665	g	0.991879	0.000631	0.004126	0.000179	0.120209	0.000202	0.001042	0.005913	0.000848
2	KM409664	0.991879	g	0.004126	0.031578	0.000187	0.525729	0.000362	0.007527	0.0447	0.005913
3	KM409668	0.000631	0.004126	h	0.991879	0.097328	0.285602	0.929168	1	0.975952	1
4	KM409689	0.004126	0.031578	0.991879	h	0.01375	0.819148	0.427142	0.999411	1	0.998042
5	KM409691	0.000179	0.000187	0.097328	0.01375	h	0.000528	0.696371	0.056116	0.009582	0.070187
6	KM409694	0.120209	0.525729	0.285602	0.819148	0.000528	g h	0.022185	0.427142	0.892081	0.366562
7	KM409695	0.000202	0.000362	0.929168	0.427142	0.696371	0.022185	h	0.819148	0.338206	0.86998
8	KM409701	0.001042	0.007527	1	0.999411	0.056116	0.427142	0.819148	h	0.996709	1
9	KM409702	0.005913	0.0447	0.975952	1	0.009582	0.892081	0.338206	0.996709	h	0.991879
10	KM409703	0.000848	0.005913	1	0.998042	0.070187	0.366562	0.86998	1	0.991879	h

Table B. 45: Descriptive statistics of the *Weissella* species at different salt (NaCl) concentrations.

Descriptive Statistics_ Salinity (*Weissella* species)											
Level	N	**0%**					**3.5 %**				
Factor		Mean	Std.Dev.	Std.Err	-95.00%	95.00%	Mean	Std.Dev.	Std.Err	-95.00%	95.00%
Total	6	0.10767	0.00804	0.00328	0.09923	0.11611	0.05967	0.03849	0.01571	0.01928	0.10005
KM409656	3	0.109	0.00872	0.00503	0.08734	0.13066	0.04067	0.05082	0.02934	-0.0856	0.1669
KM409657	3	0.10633	0.00896	0.00518	0.08407	0.1286	0.07867	0.00611	0.00353	0.06349	0.09385
Level of	N	**5%**					**6.5 %**				
Factor		Mean	Std.Dev.	Std.Err	-95.00%	95.00%	Mean	Std.Dev.	Std.Err	-95.00%	95.00%
Total	6	0.06283	0.00752	0.00307	0.05494	0.07073	-0.0045	0.00267	0.00109	-0.0073	-0.0017
KM409656	3	0.06133	0.00862	0.00498	0.03992	0.08275	-0.0047	0.00231	0.00133	-0.0104	0.00107
KM409657	3	0.06433	0.00777	0.00449	0.04504	0.08363	-0.0043	0.00351	0.00203	-0.0131	0.00439

Table B. 46: Tukey HSD analysis for *Weissella* species at different salt (NaCl) concentrations.

Cell No.	Tukey HSD test; variable 0 % Approximate Probabilities for Post Hoc Tests Error: Between MS = ,00008, df = 4,0000		
	isolates	KM409656	KM409657
		0.109	0.10633
1	KM409656	a	0.73073
2	KM409657	0.73073	a

Cell No.	Tukey HSD test; variable 3.5 % Approximate Probabilities for Post Hoc Tests Error: Between MS = ,00131, df = 4,0000		
	isolates	KM409656	KM409657
		0.04067	0.07867
1	KM409656	b	0.26808
2	KM409657	0.26808	b

Cell No.	Tukey HSD test; variable 5 % Approximate Probabilities for Post Hoc Tests Error: Between MS = ,00007, df = 4,0000		
	isolates	KM409656	KM409657
		0.06133	0.06433
1	KM409656	c	0.677685
2	KM409657	0.677685	c

Cell No.	Tukey HSD test; variable 6.5 % Approximate Probabilities for Post Hoc Tests Error: Between MS = ,00001, df = 4,0000		
	isolates	KM409656	KM409657
		-0.0047	-0.0043
1	KM409656	d	0.897544
2	KM409657	0.897544	d

Table B. 47: Descriptive statistics of the *C. maltaromaticum* sp. at different salt (NaCl) concentrations.

Descriptive Statistics_ Salinity (*C. maltaromaticum*)											
Level	N	**0%**					**3.5 %**				
Factor		Mean	Std.Dev.	Std.Err	-95,00%	95,00%	Mean	Std.Dev.	Std.Err	-95,00%	95,00%
Total	9	0,27967	0,06094	0,02031	0,23283	0,32651	0,18422	0,03171	0,01057	0,15985	0,2086
KM409658	3	0,20933	0,04441	0,02564	0,09901	0,31966	0,161	0,00656	0,00379	0,14471	0,17729
KM409660	3	0,30933	0,04013	0,02317	0,20965	0,40902	0,16733	0,00306	0,00176	0,15974	0,17492
KM409659	3	0,32033	0,00702	0,00406	0,30289	0,33778	0,22433	0,0179	0,01033	0,17987	0,26879
Level of	N	**5%**					**6.5 %**				
Factor		Mean	Std.Dev.	Std.Err	-95,00%	95,00%	Mean	Std.Dev.	Std.Err	-95,00%	95,00%
Total	9	0,17167	0,0665	0,02217	0,12055	0,22279	0,08378	0,04757	0,01586	0,04722	0,12034
KM409658	3	0,122	0,009	0,0052	0,09964	0,14436	0,038	0,00721	0,00416	0,02009	0,05591
KM409660	3	0,258	0,01308	0,00755	0,22552	0,29048	0,08467	0,05229	0,03019	-0,0452	0,21456
KM409659	3	0,135	0,02326	0,01343	0,07722	0,19278	0,12867	0,00982	0,00567	0,10429	0,15305

Table B. 48: Tukey HSD analysis for *C. maltaromaticum* sp. at different salt (NaCl) concentrations.

Cell No.	**Tukey HSD test; variable Nacl 0 %** Approximate Probabilities for Post Hoc Tests Error: Between MS = ,00121, df = 6,0000			
	isolates	KM409658	KM409660	KM409659
		0,20933	0,30933	0,32033
1	KM409658	a	0,029128	0,018692
2	KM409660	0,029128	b	0,92183
3	KM409659	0,018692	0,92183	b

Cell No.	**Tukey HSD test; variable Nacl 3.5 %** Approximate Probabilities for Post Hoc Tests Error: Between MS = ,00012, df = 6,0000			
	isolates	KM409658	KM409660	KM409659
		0,161	0,16733	0,22433
1	KM409658	c	0,774625	0,001246
2	KM409660	0,774625	c	0,002043
3	KM409659	0,001246	0,002043	d

Cell No.	**Tukey HSD test; variable Nacl 5 %** Approximate Probabilities for Post Hoc Tests Error: Between MS = ,00026, df = 6,0000			
	isolates	KM409658	KM409660	KM409659
		0,122	0,258	0,135
1	KM409658	e	0,000324	0,615193
2	KM409660	0,000324	f	0,000416
3	KM409659	0,615193	0,000416	e

Cell No.	**Tukey HSD test; variable Nacl 6.5 %** Approximate Probabilities for Post Hoc Tests Error: Between MS = ,00096, df = 6,0000			
	isolates	KM409658	KM409660	KM409659
		0,038	0,08467	0,12867
1	KM409658	g	0,234789	0,027084
2	KM409660	0,234789	g h	0,2674
3	KM409659	0,027084	0,2674	h

ELISA results

Table B. 49: ELISA absorbance results for Lesotho isolates.

Isolates	Vaccination year	KM409665 (13.02.2012)	KM409663 (11.03.2010)	KM409673 (22.10.2009)	KM409680 (02.03.2009)	KM409682 (07.01.2008)	KM409689 (17.04.2009)
2008							
KM409668 (17.01.2008)		3,68	106,77	43,4	51,84	129,74	86,01
2009							
KM659862 (15.01.2009)		3,82	73,48	27,69	20,19	157,07	99,52
KM409686 (15.01.2009)		3,48	73,3	96,42	14,04	147,36	97,65
KM409687 (15.01.2009)		3,41	73,51	39,02	14,79	174,22	105,08
KM409688 (15.01.2009)		4,64	74,67	26,83	21,76	158,77	103,09
KM409698 (19.01.2009)		3,45	90,07	55,26	16,25	164,21	85,01
KM409699 (19.01.2009)		3,72	61,82	38,16	19,38	181,37	70,01
KM409700 (19.01.2009)		3,79	82,05	60,29	16,79	226,48	56,71
KM409689 (17.04.2009)	2009 & 2010	3,68	2,79	20,36	6,05	131,13	100
KM409701 (17.04.2009)	2009	3,55	110,33	141,64	15,82	177,48	55,11
KM409702 (17.04.2009)	2009	3,89	95,50	52,81	17,01	177,56	80,57
2010							
KM409661 (23.04.2010)		3,41	84,42	73,33	31,32	103,73	69,80
2011							
KM409694 (11.03.2011)	2011, 2012 & 2013	3,96	12,15	28,01	21,60	108,07	85,83
KM409695 (11.03.2011)	2011	3,17	91,31	75,68	6,70	142,78	112,60
KM409703 (11.03.2011)	2011, 2012 & 2013	3,38	70,93	60,34	14,96	186,10	77,31
KM409704 (11.03.2011)		3,24	84,39	54,09	16,95	88,66	40,11
2012							
KM409690 (18.01.2012)		4,20	91,86	53,29	21,49	99,15	73,92
KM409691 (18.01.2012)	2012	4,67	83,48	48,05	6,59	141,69	77,18
KM409664 (13.02.2012)		4,13	4,89	7,80	19,65	61,88	35,85
Lactococcus sp KM409665 (13.02.2012)		100,00	2,89	8,93	5,29	6,29	4,09

Table B. 50: ELISA absorbance results for South Africa isolates.

Isolates	Vaccination year	KM409665 (13.02.2012)	KM409663 (11.03.2010)	KM409673 (22.10.2009)	KM409680 (02.03.2009)	KM409682 (07.01.2008)	KM409689 (17.04.2009)
	2006						
KM409685 (11.07.2006)		4,23	7,11	7,00	36,18	98,45	72,71
	2007						
KM409683 (13.12.2007)		3,72	103,68	58,90	20,25	100,62	131,12
	2008						
KM409682 (07.01.2008)		4,20	5,53	8,18	21,38	100,00	30,73
KM409705 (25.08.2008)		3,82	5,04	12,61	66,68	53,26	22,29
KM409696 (22.09.2008)		3,89	23,72	8,28	42,17	64,75	17,73
KM409675 (19.11.2008)		5,66	13,37	9,14	28,56	96,51	57,67
KM409676 (19.11.2008)		4,54	92,16	53,23	26,89	103,26	111,65
KM409677 (01.12.2008)		4,33	109,90	50,13	29,00	92,16	96,13
KM409678 (01.12.2008)		4,09	94,26	47,30	28,83	99,38	92,66
KM409679 (01.12.2008)		4,16	92,16	47,78	25,70	96,51	89,22
KM409667 (26.06.2008)		4,13	90,70	54,89	36,83	121,74	73,71
KM409684 (26.06.2008)		3,86	106,14	55,32	49,24	190,06	132,42
KM409706 (05.09.2008)		3,86	84,36	46,66	16,95	87,81	50,54
KM409707 (05.09.2008)		4,13	81,71	98,72	16,52	82,45	35,46
	2009						
KM409680 (02.03.2009)		4,91	3,65	7,06	100,00	62,27	61,23
KM409673 (22.10.2009)		3,58	79,98	100,00	8,26	119,18	81,14
KM409674 (22.10.2009)		4,54	105,13	64,14	29,05	126,16	87,05
KM409670 (19.03.2009)		3,75	89,88	40,25	29,97	119,57	79,62
KM409671 (19.03.2009)		3,65	85,27	60,82	27,00	121,51	80,75
KM409669 (25.03.2009)		3,51	89,31	18,97	16,95	115,76	72,14
KM409672 (21.10.2009)		4,40	87,88	60,50	23,76	113,35	75,32
	2010						
KM409666 (22.01.2010)		3,75	80,16	36,77	32,02	111,57	68,14
KM409662 (11.03.2010)		4,03	83,87	64,78	37,85	105,36	67,23
KM409663 (11.03.2010)		3,62	100,00	122,34	6,43	108,00	75,01
KM409681 (30.03.2010)		3,96	99,21	72,21	4,70	94,25	61,84
KM659863 (17.08.2010)		3,79	79,43	124,27	18,36	155,90	124,29
	2011						
KM409697 (21.12.2011)		3,58	4,34	9,25	55,45	57,84	27,47
KM409692 (06.12.2011)		4,23	12,67	63,92	22,52	109,01	75,36
KM409693 (06.12.2011)		4,91	13,37	53,02	23,76	97,44	77,05
	2012						
KM659864 (08.02.2012)		3,75	8,41	6,15	67,49	92,16	50,76